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# JOURNAL OF DAIRY SCIENCE

OFFICIAL ORGAN OF THE
AMERICAN DAIRY SCIENCE ASSOCIATION

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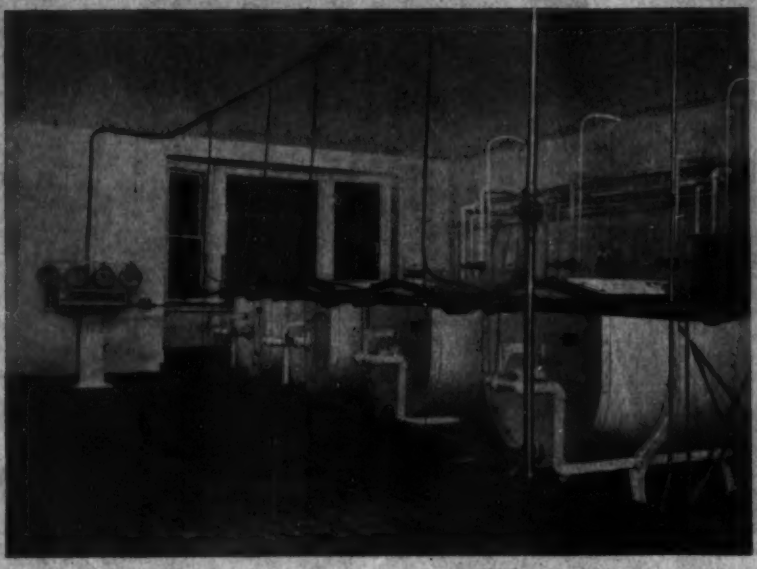

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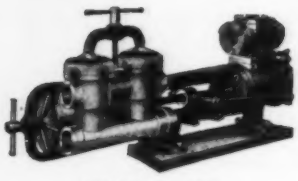

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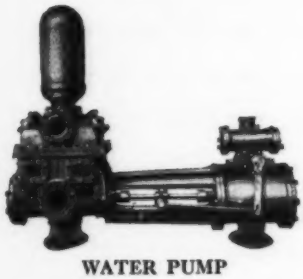

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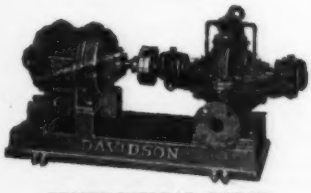


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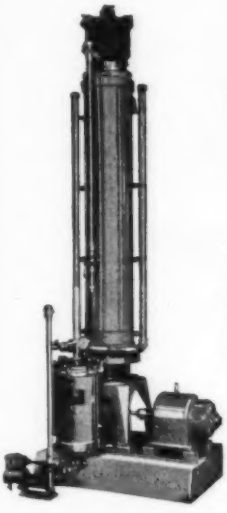

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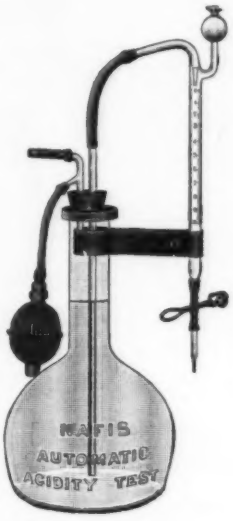


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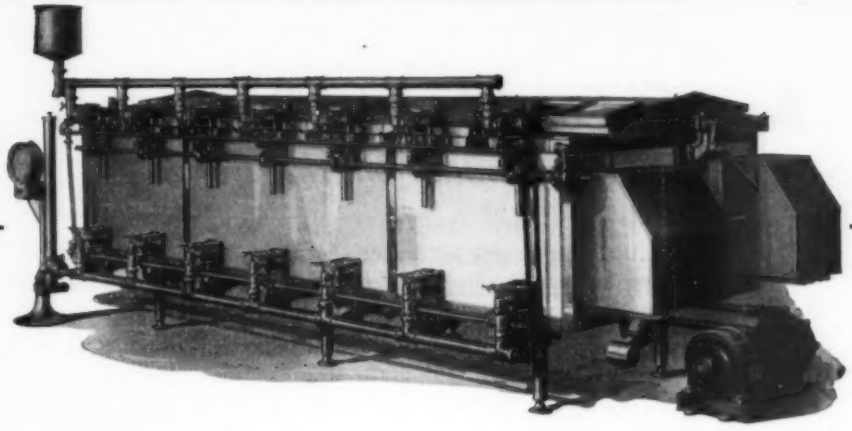

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# THE MINERAL METABOLISM OF DAIRY COWS AS AFFECTED BY DISTILLED WATER AND PREVIOUS FEEDING*

C. F. MONROE AND A. E. PERKINS

*Ohio Agricultural Experiment Station, Wooster, Ohio*

The results of our former mineral metabolism experiments (1) have raised some questions in our minds. These results differed from those of other investigators, principally, in that our cows showed a better utilization of calcium. We failed to find any sensational negative balances for this element which some have believed were inevitable with cows producing as these did, 30 pounds or more of milk per day.

We were reluctant to think, however, that our results were inconsistent with the history of the animals. In our previous discussion (1) we summarized the situation as follows:

> As a matter of fact, we are convinced, after examining their milk records in the several lactation periods, that the assimilation of calcium by these cows must have been more favorable than most of the mineral metabolism experiments have shown; for, if this had not been true, either the milk production would have been considerably less or the animals themselves would have come to disaster.

## COMPARISON OF RESULTS

In comparing our experiments with others relating to this same subject we were impressed with certain factors which may have caused the more favorable balances for our cows. These factors are briefly discussed in the following paragraphs.

### *1. Vitamin content of hay*

The quality of the clover hay fed in our experiments deserves consideration, especially in view of the evidence submitted by

* Published with the approval of the Director of the Ohio Agricultural Experiment Station. Received for publication April 10, 1925.

Hart and associates (2). These workers observed more favorable calcium balances when (3) feeding alfalfa hay cured under caps than when feeding (4) alfalfa hay cured by four days' exposure to the sun and weather while in the window. They suggest that curing hay without undue exposure to the sun and weather aids in preserving the unknown factors affecting calcium assimilation (3). The clover hay used in our experiments was of choice quality, and had retained remarkably well its original color. It was cured in the sun for two days; then spread out on the barn floor and allowed to dry thoroughly. When fed this hay was comparatively fresh and had not passed through the sweat of the mow.

*2. Effect of direct sunlight*

Our previous experiments were conducted in the summer with most of the windows in the barn open. Immediately back of the cows were four open windows, allowing the direct sunshine to fall on the animals during part of the day. Hart and associates (5) have shown that the utilization of calcium and phosphorus by milking goats was increased by exposing the animals for twenty minutes daily to the emanations from a quartz mercury vapor lamp. As direct sunshine has been shown (6) to possess antirachitic properties similar to those exhibited by the rays from the quartz mercury vapor lamp, it is probable that this factor had a beneficial influence on the calcium and phosphorus utilization by our cows.

*3. Favorable influence of beet pulp.*

The liberal use of beet pulp in our rations may well be listed among these factors. Gaessler and McCandlish (7) have noted positive calcium balances when beet pulp was a constituent of the ration, but they have attributed the favorable balances to the quality of the alfalfa hay fed at the same time. However, we do know that beet pulp has met with much favor as a feed for milking cows, and, owing to its comparatively high calcium content, it could be considered a mineral supplement. In our low protein rations approximately one-half of the calcium was supplied by beet pulp.

### *4. Effect of natural water*

In both of our experiments, natural, or tap water, was given the cows in preference to distilled water. This was done in order to avoid a change in the rations and to make the experimental conditions as nearly similar to the usual conditions as possible. In nearly all of the mineral metabolism experiments distilled water has been used. The kind of water used may or may not be a matter of great importance. As we had no knowledge of any definite experimental data regarding this factor, we thought it worthy of consideration.

### *5. History of cows*

Another very important factor was the history of the cows used in our work. These cows had never been allowed access to pasture or any other green feed. (They had received silage; but this is not generally considered a green feed). If it is true, as hypethecated by Forbes (8), that fresh, green grass (green roughage) with its "fresh grass vitamin" is necessary to the building up of large mineral reserves, then the status of our dry-feed cows regarding mineral reserves would be different from that of cows which had been allowed access to pasture or soilage. Presumably, most of the cows used in the other mineral metabolism experiments belonged to the latter class. The question naturally follows then, would this difference in the previous management of the cows lead to a difference in mineral balances.

## OBJECT

In this, our third mineral metabolism experiment, we have confined ourselves to a study of the last two factors discussed. The object of this work has been, therefore, twofold: First, to compare the balances of cows given distilled water with those of cows given natural or tap water, and, second, to compare the balances of cows that have been fed continually on winter rations with those of cows that have been allowed pasture in summer.

With these objects in view we planned this experiment in such a way as to either eliminate or equalize the effects of the other

three factors, namely, the quality of the clover hay, direct sunshine, and beet pulp. This was accomplished by: First, feeding timothy hay as the roughage of the ration, and, second, eliminating direct sunlight by keeping the windows in the barn closed (this experiment was conducted in the winter). Third, equalizing the amounts of beet pulp in the rations.

## EXPERIMENTAL

### *Cows*

Four Holstein-Friesian cows were used in the work. Two of them, 146 and 154 were from the group of dry-feed cows and had been used in each of the former experiments. The other two, 163 and 203, were from the regular herd which had been pastured the preceding summer. In designating the two groups, we shall hereafter refer to the former as dry-feed cows and to the latter as regular herd cows.

Table 1 gives some of the more important facts concerning the cows. At the beginning of the experiment, cow 154 was in the fifth month of gestation; while 146 and 203 were in the second and first months, respectively. Cow 163 was bred during the interval between the periods of the experiment. Cow 203 freshened 343 days prior to the test; 154, 263 days; 146, 147 days; and 163, 118 days.

The average daily milk production during the experiment varied from 29 pounds for cow 154 to 32 pounds for 163; cows 146 and 203 taking intermediate positions with productions of 30 and 29.8 pounds respectively. It may be observed that these milk yields correspond very closely to the average daily yields for the entire lactations.

The average live weights for the six days at the beginning and end of the experiment are also given in table 1. Almost insignificant gains in weight are shown for cows 146 and 203. Cow 154 showed a significant gain of 51 pounds. No. 163 lost 26 pounds.

### *Experimental procedure*

The general methods used in this work were similar to those used in our former experiments (1).

The experiment consisted of two balance periods of eighteen days each, separated by an interval of twelve days. The only difference in the procedure in the two periods was in the nature of the water received by the cows, distilled water being given to one cow of each group, while the other cow of the group received natural water. This procedure was then reversed in the second period.

TABLE 1

*Data concerning cows used*

| | cow 146 | cow 154 | cow 163 | cow 203 |
|---|---|---|---|---|
| Breed | H. F. (Grade) | H. F. | H. F. | H. F. |
| Grouping | Dry-feed | Dry-feed | Regular herd | Regular herd |
| Age, years | 8.5 | 8.5 | 7.5 | 6 |
| Previous lactations | 5 | 5 | 4 | 1 |
| Freshened prior to test | 7/8/23 | 3/12/23 | 8/7/23 | 1/23/23 |
| Day of lactation at start of test | 147 | 263 | 118 | 343 |
| Live weight (average first six days of test) | 1,203 | 1,301 | 1,304 | 1,126 |
| Live weight (average last six days of test) | 1,206 | 1,352 | 1,278 | 1,130 |
| Date bred | 11/12/23 | 7/7/23 | 1/2/24 | 10/21/23 |
| Month of gestation (start of test) | 1 | 5 | 0 | 2 |
| Average daily milk production for test, pounds | 30 | 29 | 32 | 29.8 |
| Average daily milk production for lactation, pounds | 29.4 | 32 | 28 | 27.3 |
| Days in milk (lactation period) | 298 | 366 | 340 | 509 |

Each of these periods was divided into three 6-day sub-periods. Composite samples of milk, of urine, and of feces were made for each of the sub-periods. These composite samples were analyzed for the elements under consideration. Details for the sub-periods are not given here, because they showed no marked variation from the average daily figures here presented. Our purpose in basing the results on a number of samples rather than on one composite sample for each product was to reduce as much as possible the chances for error.

*Sampling feeds and water*

The mineral and nitrogen analyses of the feeds and water are listed in table 2. Quantities of each of the grains and beet pulp sufficient for the entire experiment, were individually mixed, sacked and divided into two parts. One part was used in the first preliminary feeding and period I; The other was reserved for the second preliminary feeding and period II. Samples of each part were taken when the feeds were weighed for the individual rations. Separate analyses of the two sets of samples were made, but the results were so nearly identical that only one value is used.

TABLE 2

*Composition of feed—per cent (as weighed for rations)*

| FEED | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|
| Timothy hay, first period | 0.098 | 0.287 | 0.076 | 0.594 |
| Timothy hay, second period | 0.113 | 0.296 | 0.089 | 0.675 |
| Beet pulp | 0.081 | 0.696 | 0.290 | 1.410 |
| Corn | 0.222 | 0.008 | 0.110 | 1.490 |
| Bran | 1.616 | 0.127 | 0.621 | 2.360 |
| Oats | 0.399 | 0.081 | 0.147 | 2.030 |
| Molasses | 0.075 | 0.604 | 0.208 | 0.430 |
| Water, first period | | 0.0052 | 0.0019 | 0.00012 |
| Water, second period | | 0.0035 | 0.0012 | 0.00014 |

All the cane molasses used came from the same barrel. The analyses of the samples for each period were practically the same.

It was impossible to mix the timothy hay thoroughly because of its great bulk and the possbbility of shattering the heads. To compensate for this, we have taken a larger number of subsamples. The difference in the composition of the hay used in the two periods is attributed largely to a greater contamination with clover in that fed during period II. The hay was chopped by running it through a silage cutter. This hay was of poor quality and had evidently been over-ripe when cut. It contained some coarse woody stems of weeds which the cows refused to eat. However, it was the best timothy hay obtainable at that time.

Water samples were taken daily. Aliquot samples representing the 6 sub-periods for each cow were made from these samples.

The distilled water was sampled and analyzed for its mineral content by the same methods used for tap water, the results in all cases being negligible. The natural water, though supposed to be entirely of deep well origin, contained a considerable amount of sediment following storms. Its mineral content was slightly lower in period II than in period I.

### *Rations*

The rations fed during the experiment are given in table 3. These rations were identical in composition and amount, with the one exception of a 5 per cent reduction for cow 203 during

TABLE 3

*Mineral and nitrogen content of daily ration (grams)*

| FEED | AMOUNT* | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|---|
| | *pounds* | | | | | |
| Timothy hay | 10.5 | 4,762 | 4.7 | 13.7 | 3.6 | 28.3 |
| Beet pulp | 9.8 | 4,464 | 3.6 | 31.1 | 12.9 | 63.0 |
| Corn | 6.5 | 2,948 | 6.6 | 0.2 | 3.3 | 43.9 |
| Bran | 3.25 | 1,474 | 23.8 | 1.9 | 9.2 | 34.8 |
| Oats | 3.25 | 1,474 | 5.9 | 1.2 | 2.2 | 29.9 |
| Molasses | 1.3 | 590 | 0.4 | 3.5 | 1.2 | 2.5 |
| Total | | | 45.0 | 51.6 | 32.4 | 202.4 |

* Ration for no. 203 was 5 per cent less than this during period II.

the second period. She consumed all of her feed during period I, but showed signs of being slightly overfed, as judged by her slow eating. This cow was the smallest of the experimental animals. No difficulty was experienced in getting the cows to consume completely the edible part of the rations. There was some refuse, which consisted largely of coarse weed stems found in the hay. This was carefully weighed and later analyzed. When computing the quantity of mineral matter contained in the refuse it was found that it did not amount to 0.1 gram a day, hence these figures are omitted in this discussion.

The molasses was diluted with approximately an equal weight

of water and sprinkled over the hay. This seemed to be very necessary in coaxing the cows to eat the hay. The dried sugar-beet pulp used in the rations was fed in the wet condition, having been soaked in 3 times its weight of water. The cows were extremely eager for the beet pulp. Yellow corn of good quality was fed in a finely ground condition. The oats were also ground. Water was given the cows "ad libitum." Nearly equal amounts of the two types of water were consumed in the course of the experiment. The slight difference of 0.9 per cent favored a larger consumption of natural water.

The mineral and nitrogen content of the daily rations is also given in table 3. The importance of beet pulp in the rations is here shown. This feed furnished approximately 60 per cent of the calcium, 40 per cent of the magnesium and 31 per cent of the nitrogen of the ration. Beet pulp contains very small amounts of phosphorus; 52 per cent of the phosphorus was supplied by the wheat bran.

Rations similar to those fed during the test had been fed to the two dry-feed cows (146 and 154) since their last freshening. The other two cows, (163 and 203), taken from the regular herd,[1] were fed these rations approximately 3 weeks prior to the beginning of the experiment. The cows were placed in the experimental stalls one week previous to the beginning of the experiment proper. Feeding of the specially weighed rations started at this time. The actual routine of the experiment had been in progress three days before the samples of milk and excreta were taken.

The data here presented were taken from December 5 to 23, 1923, for period I; and from January 4 to 22, 1924 for period II.

## DISCUSSION

In tables 4 to 7, inclusive, will be found the detailed figures relating to the mineral and nitrogen balances.

[1] Ration: Grain mix (4 pints ground corn, 1 pint bran, and 1 pint oilmeal), 1 pound for every 3 pounds of milk produced; alfalfa hay, approximately 10 pounds per day.

TABLE 4

*Average daily balances of minerals and nitrogen (grams)*

Cow 146 (Dry-feed)

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| *Period I. Distilled water period (18 days)* | | | | | |
| Intake | | | | | |
| Feed | 15,712 | 45.0 | 51.6 | 32.4 | 202.4 |
| Water | 56,378 | | | | |
| Total intake | | 45.0 | 51.6 | 32.4 | 202.4 |
| Outgo | | | | | |
| Milk | 14,064 | 14.0 | 17.0 | 1.7 | 72.5 |
| Urine, cc | 6,771 | | 5.1 | 6.6 | 33.4 |
| Feces | 5,487 | 28.3 | 35.0 | 23.1 | 94.4 |
| Total outgo | | 42.3 | 57.1 | 31.4 | 200.3 |
| Balance | | +2.7 | −5.5 | +1.0 | +2.1 |
| *Period II. Natural Water Period (18 days)* | | | | | |
| Intake | | | | | |
| Feed | 15,712 | 45.7 | 52.0 | 33.0 | 206.3 |
| Water | 52,633 | | 1.9 | 0.7 | 0.1 |
| Total intake | | 45.7 | 53.9 | 33.7 | 206.4 |
| Outgo | | | | | |
| Milk | 13,149 | 13.0 | 15.5 | 1.4 | 67.2 |
| Urine. cc | 5,355 | | 4.2 | 6.8 | 31.6 |
| Feces,. | 5,518 | 27.8 | 36.8 | 24.4 | 96.8 |
| Total outgo | | 40.8 | 56.5 | 32.6 | 195.6 |
| Balance | | +4.9 | −2.6 | +1.1 | +10.8 |
| *Average for both periods (36 days)* | | | | | |
| Intake | | | | | |
| Feed | 15,712 | 45.4 | 51.8 | 32.7 | 204.4 |
| Water | 54,506 | | 1.0 | 0.4 | |
| Total intake | | 45.4 | 52.8 | 33.1 | 204.4 |

TABLE 4—*Continued*

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| | | | Outgo | | |
| Milk | 13,607 | 13.5 | 16.3 | 1.6 | 69.9 |
| Urine. cc. | 6,063 | | 4.7 | 6.7 | 32.5 |
| Feces | 5,503 | 28.0 | 35.9 | 23.8 | 95.6 |
| Total outgo | | 41.5 | 56.9 | 32.1 | 198.0 |
| Balance | | +3.9 | −4.1 | +1.0 | +6.4 |

TABLE 5

*Average daily balances of minerals and nitrogen (grams)*

Cow 154 (Dry-feed)

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| | | *Period I. Natural Water Period (18 days)* | | | |
| | | Intake | | | |
| Feed | 15,712 | 45.0 | 51.6 | 32.4 | 202.4 |
| Water | 52,317 | | 2.6 | 1.0 | 0.1 |
| Total intake | | 45.0 | 54.2 | 33.4 | 202.5 |
| | | Outgo | | | |
| Milk | 13,957 | 14.3 | 15.4 | 1.8 | 70.8 |
| Urine, cc | 8,549 | | 4.4 | 6.4 | 33.4 |
| Feces | 5,128 | 28.7 | 37.8 | 23.1 | 91.6 |
| Total outgo | | 43.0 | 57.6 | 31.3 | 195.8 |
| Balance | | +2.0 | −3.4 | +2.1 | +6.7 |
| | | *Period II. Distilled water period (18 days)* | | | |
| | | Intake | | | |
| Feed | 15,712 | 45.7 | 52.0 | 33.0 | 206.3 |
| Water | 50,650 | | | | |
| Total intake | | 45.7 | 52.0 | 33.0 | 206.3 |

TABLE 5—*Continued*

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| | | Outgo | | | |
| Milk | 12,398 | 12.1 | 14.0 | 1.6 | 63.2 |
| Urine, cc. | 6,798 | | 3.9 | 5.9 | 33.3 |
| Feces | 5,437 | 26.7 | 32.7 | 23.3 | 97.1 |
| Total outgo | | 38.8 | 50.6 | 30.8 | 193.6 |
| Balance | | +6.9 | +1.4 | +2.2 | +12.7 |
| | | *Average for both periods (36 days)* | | | |
| | | Intake | | | |
| Feed | 15,712 | 45.4 | 51.8 | 32.7 | 204.4 |
| Water | 51,484 | | 1.3 | 0.5 | |
| Total intake | | 45.4 | 53.1 | 33.2 | 204.4 |
| | | Outgo | | | |
| Milk | 13,178 | 13.2 | 14.7 | 1.7 | 67.0 |
| Urine, cc. | 7,674 | | 4.2 | 6.2 | 33.4 |
| Feces | 5,283 | 27.7 | 35.3 | 23.2 | 94.4 |
| Total outgo | | 40.9 | 54.2 | 31.1 | 194.8 |
| Balance | | +4.5 | −1.1 | +2.1 | +9.6 |

TABLE 6

*Average daily balances of minerals and nitrogen (grams)*

Cow 163 (regular herd)

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| | | *Period I. Distilled water period (18 days)* | | | |
| | | Intake | | | |
| Feed | 15,712 | 45.0 | 51.6 | 32.4 | 202.4 |
| Water | 53,450 | | | | |
| Total intake | | 45.0 | 51.6 | 32.4 | 202.4 |

TABLE 6—*Continued*

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| **Outgo** | | | | | |
| Milk | 14,816 | 15.1 | 18.1 | 1.8 | 76.1 |
| Urine, cc | 8,200 | | 7.0 | 8.1 | 39.9 |
| Feces | 4,819 | 30.7 | 36.2 | 21.5 | 92.4 |
| Total outgo | | 45.8 | 61.3 | 31.4 | 208.4 |
| Balance | | −0.8 | −9.7 | +1.0 | −6.0 |
| *Period II. Natural water period (18 days)* | | | | | |
| **Intake** | | | | | |
| Feed | 15,712 | 45.7 | 52.0 | 33.0 | 206.3 |
| Water | 54,933 | | 1.9 | 0.7 | 0.1 |
| Total intake | | 45.7 | 53.9 | 33.7 | 206.4 |
| **Outgo** | | | | | |
| Milk | 14,179 | 14.3 | 17.2 | 1.7 | 73.3 |
| Urine, cc | 6,448 | | 7.2 | 8.4 | 37.7 |
| Feces | 5,244 | 27.9 | 37.3 | 21.6 | 97.0 |
| Total outgo | | 42.2 | 61.7 | 31.7 | 208.0 |
| Balance | | +3.5 | −7.8 | +2.0 | −1.6 |
| *Average for both periods (36 days)* | | | | | |
| **Intake** | | | | | |
| Feed | 15,712 | 45.4 | 51.8 | 32.7 | 204.4 |
| Water | 54,192 | | 1.0 | 0.4 | |
| Total intake | | 45.4 | 52.8 | 33.1 | 204.4 |
| **Outgo** | | | | | |
| Milk | 14,498 | 14.7 | 17.7 | 1.8 | 74.7 |
| Urine, cc | 7,324 | | 7.1 | 8.3 | 38.8 |
| Feces | 5,032 | 29.3 | 36.8 | 21.6 | 94.7 |
| Total outgo | | 44.0 | 61.6 | 31.7 | 208.2 |
| Balance | | +1.4 | −8.8 | +1.4 | −3.8 |

TABLE 7

*Average daily balances of minerals and nitrogen (grams)*

Cow 203 (regular herd)

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| *Period I. Natural water period (18 days)* | | | | | |
| Intake | | | | | |
| Feed | 15,712 | 45.0 | 51.6 | 32.4 | 202.4 |
| Water | 55,495 | | 2.9 | 1.1 | 0.1 |
| Total intake | | 45.0 | 54.5 | 33.5 | 202.5 |
| Outgo | | | | | |
| Milk | 13,464 | 13.5 | 15.5 | 1.9 | 73.5 |
| Urine, cc. | 4,088 | | 5.8 | 8.6 | 31.7 |
| Feces | 5,601 | 30.4 | 41.3 | 21.8 | 100.9 |
| Total outgo | | 43.9 | 62.6 | 32.3 | 206.1 |
| Balance | | +1.1 | −8.1 | +1.2 | −3.6 |
| *Period II. Distilled water period (18 days)* | | | | | |
| Intake | | | | | |
| Feed | 14,929 | 43.4 | 49.4 | 31.4 | 196.0 |
| Water | 52,833 | | | | |
| Total intake | | 43.4 | 49.4 | 31.4 | 196.0 |
| Outgo | | | | | |
| Milk | 13,557 | 13.5 | 16.0 | 2.0 | 71.7 |
| Urine, cc. | 3,960 | | 6.2 | 7.7 | 29.1 |
| Feces | 5,603 | 29.1 | 36.1 | 22.2 | 95.0 |
| Total outgo | | 42.6 | 58.3 | 31.9 | 195.8 |
| Balance | | +0.8 | −8.9 | −0.5 | +0.2 |
| *Average for both periods (36 days)* | | | | | |
| Intake | | | | | |
| Feed | 15,320 | 44.2 | 50.5 | 31.9 | 199.2 |
| Water | 54,164 | | 1.5 | 0.6 | |
| Total intake | | 44.2 | 52.0 | 32.5 | 199.2 |

TABLE 7—*Continued*

| | AMOUNT | PHOSPHORUS | CALCIUM | MAGNESIUM | NITROGEN |
|---|---|---|---|---|---|
| | | | Outgo | | |
| Milk | 13,510 | 13.5 | 15.8 | 2.0 | 72.6 |
| Urine, cc. | 4,024 | | 6.0 | 8.1 | 30.1 |
| Feces | 5,602 | 29.8 | 38.7 | 22.0 | 98.0 |
| Total outgo | | 43.3 | 60.5 | 32.1 | 200.7 |
| Balance | | +0.9 | −8.5 | +0.4 | −1.5 |

*Phosphorus balances*

Retention of this element was shown in 7 cases out of 8; the one negative balance, that of cow 163, period I, amounted to only 0.8 gram. The largest storage of phosphorus occurred in the balance for cow 154 during the second period: this daily retention of 6.9 grams was accompanied by a storage of all the elements studied, together with a gain in weight and decline in milk production.

The type of water given the cows apparently had little influence on the phosphorus balances. Greater quantities of this element were retained by 3 of the 4 cows during period II than during period I. In the case of the fourth cow the decrease in positive balance, amounting to 0.3 gram, does not seem significant in view of the fact that her ration, hence her phosphorus intake, had been reduced. As two of the cows received natural water during the second period, and the other two distilled water (both sets having been reversed in this respect from the first period), it is evident that the phosphorus balances show no relationship to the type of water used.

We believe that the increased positive balances for the 3 cows during period II can be explained largely by the decrease in milk production during this period. The increase in milk production by the fourth cow was accompanied by a decrease in storage. It must also be remembered that the ration of this cow (203, period II) had been slightly reduced. Data bearing on the rela-

tionship between the changes in milk production and the phosphorus balances are given in table 8.

A comparison of the phosphorus balances for the two groups shows that, for the entire 36 days of the experiment, the dry-feed group had larger positive balances than the regular herd group. The average daily retentions by the dry-feed cows, 146 and 154, were 3.9 and 4.5 grams, respectively; while those of the regular herd cows, 163 and 203, were 1.4 and 0.9 gram, respectively. It is true that the milk production for the latter group slightly exceeded that for the former; but this difference was not sufficient to account for the variation in the balances.

TABLE 8

| COW | DECLINE IN MILK YIELD | INCREASE IN PHOSPHORUS BALANCE | RATIO |
|---|---|---|---|
| | *grams* | *grams* | |
| 146 | 915 | 2.2 | 1:0.0024 |
| 154 | 1,559 | 4.9 | 1:0.0031 |
| 163 | 637 | 4.3 | 1:0.0067 |
| 203 | 93 (Increase) | 0.3 (Decrease) | 1:0.0032 |

## CALCIUM BALANCES

There was only one storage of calcium and that was for cow 154 during the second period, the average daily retention in this case being 1.4 grams. The losses varied from 2.5 grams for cow 146, period II, to 9.7 grams for 163, period I.

The calcium balances for cows 146, 154, and 163 were more favorable during the second half of the work than during the first half. Like the phosphorus balances, it seems that the general difference in the calcium balances can be very largely explained by the decline in milk production. As a matter of interest, the relationship between the change in calcium balances and the decline in milk production is presented in table 9. This table shows that for every gram decline in milk there was an increase in the calcium balance amounting to approximately 3 mgm.

When considering the nature of the water used in mineral metabolism experiments as a possible factor affecting the assimi-

lation of minerals, it was believed that the effects of the natural water would be more evident in the calcium balances than in the other mineral balances, because calcium was the element found in the largest quantities in the water. As previously indicated, the data for period II show more favorable calcium balances than those for period I, regardless of the nature of the water used. Therefore, it would seem fair to assume that the natural water had no marked effect on the utilization of this element.

For the entire thirty-six days the average daily balances were more favorable for the dry-feed group than for the regular herd group. Though the regular herd cows produced slightly more milk, the difference in milk production was not sufficient to account for the difference in calcium balances. These results

TABLE 9

| COW | DECLINE IN MILK YIELD | INCREASE IN CALCIUM BALANCE | RATIO |
|---|---|---|---|
| | *grams* | *grams* | |
| 146 | 915 | 2.9 | 1:0.0031 |
| 154 | 1,559 | 4.8 | 1:0.0031 |
| 163 | 637 | 1.9 | 1:0.0030 |
| 203 | 93 (Increase) | 0.8 (Decrease) | 1:0.0086 |

compare as follows: the dry-feed cows, 146 and 154 lost approximately 4 and 1 gram per day, respectively; while the regular herd cows, 163 and 203, each lost approximately 8.5 grams daily.

### *Magnesium balances*

Of the eight balances for this element, only one was negative, and this only to the extent of 0.5 gram daily. This loss was shown by cow 203, during the second period. Only a slight variation was shown in the 7 positive balances: the smallest storage was that of cow 146, period I, amounting to 1 gram; and the largest, that of cow 154, period II, amounting to 2.2 grams.

The type of water used had no effect on the magnesium balances. It seemingly had an influence on the outgo of this element in the urine, the average daily quantity for the individual cows being slightly increased when natural water was used.

Approximately 1 gram of magnesium was supplied daily by the water.

A comparison of the magnesium balances of the two groups for the thirty-six days does not show that any distinctive difference existed between them. It is, interesting to note the similarity in magnesium outgo in the urine of the two cows in each group. Greater amounts of this element were found in the urine of the regular herd group.

*Nitrogen balances*

The nitrogen balances were positive for the two dry-feed cows in both periods; and negative, except in one instance, for the regular herd cows. The one storage of nitrogen, in the latter group, was less than 0.5 gram per day. Variations from a 12.7 grams retention for cow 154, period II, to a 6 grams loss for cow 163, period I are shown.

The average daily data for the entire time covered by the experiment show positive balances of 6.4 and 9.6 grams for the dry-feed cows, 146 and 154; and negative balances of 3.8 and 1.5 gram for the regular herd cows, 163 and 203.

The type of water used in the ration had no apparent effect on the nitrogen balances.

This work shows that the calcium, phosphorus, and nitrogen balances are influenced to some extent at least by the previous feeding of the cows. In our opinion the difference in the previous feeding of the two groups had affected the amounts of the available elements stored in their bodies. It seems logical to assume that the dry-feed cows, which had long received these rations known to be conducive to mineral and nitrogen losses, would have smaller reserves than the regular herd cows, which had received the same rations for only three weeks previous to the experiment. (The latter group had been changed from a ration containing alfalfa hay and a more liberal protein supply.) If this assumption is correct, then the extent of the positive and negative balances was influenced to some degree by the amounts of available elements in reserve, as shown in the data here reported. In this light, we would interpret some of the large

negative balances that have been observed as indicating losses in extensive reserves rather than as proving that a fairly liberal milk production (30 pounds per day) is impossible without large losses of minerals and nitrogen, even under winter conditions.

That the dry-feed cows averaged almost 30 pounds of milk per day is shown by the records for their fourth, fifth, and sixth lactations, or those periods during which our experiments were conducted (table 10; see table 1, also for the sixth lactation). The rations received by these cows during the sixth lactation were the same as those used in this experiment. In the fourth and fifth lactations, rations containing some clover hay were given. The details of this feeding are given in our former paper (1).

TABLE 10

*Summary of milk and fat production for dry-feed cows, (fourth, fifth, and sixth periods)*

| COW | MILK PRODUCTION | FAT PRODUCTION | DAYS IN MILK | DAYS DRY | AVERAGE DAILY MILK PRODUCTION | AVERAGE WEIGHT OF CALVES |
|---|---|---|---|---|---|---|
| | *pounds* | *pounds* | | | *pounds* | *pounds* |
| 146* | 30,067 | 1,162 | 1,028 | 218 | 29.1 | 107 |
| 154 | 31,220 | 1,040 | 1,073 | 174 | 29.1 | 119 |

* Aborted seven months, calf weighing 106 pounds, in 1924.

## *Summary of discussion*

Eight balances of phosphorus, calcium, magnesium, and nitrogen are reported. Four of these balances were determined on cows that had always been restricted to dry feeds; and four on cows taken from the regular herd, which had been pastured the preceding summer. A comparison was made of the balances of cows given distilled water with those of cows given natural or deep well water. The double reversal method was used for making this comparison. All rations were otherwise identical with the exception of a 5 per cent reduction in that of one cow during the latter part of the work. The level of daily milk production was approximately 30 pounds.

The calcium balances formed the chief difference between the dry-feed and regular herd groups. Taking the entire thirty-six

days as the unit, all these balances were negative; those of the regular herd cows, amounting to approximately 8.5 grams daily, were more than double the losses sustained by the dry-feed cows.

The regular herd cows lost nitrogen at the rate of 1.5 and 3.8 grams daily; while the dry-feed cows stored 6.4 and 9.6 grams.

The phosphorus balances showed that the regular herd cows retained only 0.9 and 1.4 grams daily, while the dry-feed cows retained 3.9 and 4.5 grams daily.

No difference was found between the two groups in respect to the magnesium balances.

The type of water used in the rations had no apparent effect on the balances of any of the elements studied. The daily calcium intake from the natural water varied from 2 to 3 grams, while the magnesium intake from this source approximated 1 gram daily.

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# EFFECT OF BONE MEAL ON GROWTH OF DAIRY HEIFERS*

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The literature contains considerable data on the effect of calcium and phosphorus compounds on milking cows with reference to the milk production and the maintenance of equilibrium of these elements. The data on the effect of such compounds on the growth of dairy animals are very meager.

Hart (2) et al. report that the addition of bone meal and cod liver oil to rations made up of products of the wheat plant gave a marked improvement in the growth and reproduction of dairy cows.

Eckles (1) reports that a Jersey heifer receiving a ration low in mineral made a growth both in height and weight equal to a similar heifer receiving a ration that contained approximately three times as much calcium and phosphorus. The heifer on the low mineral ration suffered a break down in condition at the age of 18 months and it was necessary to change the ration.

Sir Arnold Theiler (3) has found that the feeding of bone meal has a very favorable effect on the gains of cattle that are grazed on native grasses in South Africa where the soil contains very little calcium and phosphorus and where lack of sufficient moisture makes conditions very unfavorable for the assimilation of these constituents by plants.

The problem of whether our common farm rations can be improved by the addition of various mineral supplements is an important one. An experiment to test the effect of adding bone meal to a ration which is often fed dairy heifers was begun at this station in December, 1922.

* Received for publication March 6, 1925.

## EXPERIMENTAL

### *Plan*

Two groups of 4 heifers each were used. The groups were fairly uniform with respect to individuality of the animals. Both groups received a grain mixture of 300 pounds of shelled corn and 200 pounds of oats. The hay given consisted chiefly of

TABLE 1

*Feed consumed by periods (December 10, 1922, to April 26, 1924, inclusive)*

Lot 1—2 ounces bone meal daily

| PERIODS 28 DAYS EACH | HEIFER 45 | | HEIFER 47 | | HEIFER 49 | | HEIFER 43 | |
|---|---|---|---|---|---|---|---|---|
| | Grain | Hay | Grain | Hay | Grain | Hay | Grain | Hay |
| | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* |
| 1 | 112 | 21 | 112 | 21 | 112 | 21 | 112 | 21 |
| 2 | 145.5 | 48 | 145 | 48 | 145 | 48 | 145 | 48 |
| 3 | 168 | 56 | 168 | 56 | 168 | 56 | 168 | 56 |
| 4 | 168 | 56 | 168 | 56 | 168 | 56 | 155 | 56 |
| 5 | 168 | 56 | 168 | 56 | 168 | 56 | 168 | 56 |
| 6 | 115 | 56 | 115 | 56 | 114 | 56 | 111.5 | 56 |
| 7 | 112 | 56 | 112 | 56 | 112 | 56 | 111 | 56 |
| 8 | 84 | 54 | 81.5 | 54 | 81.5 | 54 | 84 | 54 |
| 9 | 82.5 | 56 | 82.5 | 56 | 80.5 | 56 | 81.5 | 56 |
| 10 | 84. | 56 | 83 | 56 | 84 | 56 | 84 | 56 |
| 11 | 84 | 56 | 84 | 56 | 84 | 56 | 84 | 56 |
| 12 | 103 | 56 | 101 | 56 | 104 | 56 | 104 | 56 |
| 13 | 96 | 48 | 97 | 54 | 98 | 56 | 102 | 56 |
| 14 | Calved and | | 150 | 56 | 150 | 56 | 150 | 56 |
| 15 | ration | | 168 | 56 | 168 | 56 | 168 | 56 |
| 16 | changed | | 168 | 56 | 168 | 56 | 168 | 56 |
| 17 | | | 168 | 56 | 168 | 56 | 168 | 56 |
| 18 | | | 140 | 56 | 140 | 56 | 140 | 56 |
| Total... | 1,522 | 675 | 2,311 | 961 | 2,313 | 963 | 2,304.0 | 963 |

Johnson Grass and timothy. Both groups had access to block salt and one group received 2 ounces of steamed bone meal mixed in grain each day. Tables 1 and 2 show feed records by periods of twenty-eight days.

During the grazing season the heifers were on Bermuda, carpet grass, and dallis grass pasture. This pasture was on acid land of low lime content. Weights and measurements were taken

every twenty-eight days; although only a part of the data are presented.

### *Results*

Curves of growth of the heifers in weight and height are shown in figures 1 and 2. The feeding of 2 ounces of

TABLE 2

*Feed consumed by periods (December 10, 1922, to April 26, 1924, inclusive)*

Lot 2—no mineral

| PERIODS 28 DAYS EACH | HEIFER 46 | | HEIFER 43 | | HEIFER 41 | | HEIFER 42 | |
|---|---|---|---|---|---|---|---|---|
| | Grain | Hay | Grain | Hay | Grain | Hay | Grain | Hay |
| | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* | *pounds* |
| 1 | 112 | 21 | 112 | 21 | 112 | 21 | 112 | 21 |
| 2 | 140.5 | 48 | 145 | 48 | 137.3 | 48 | 138.5 | 48 |
| 3 | 168 | 56 | 168 | 56 | 125.5 | 56 | 168 | 56 |
| 4 | 168 | 56 | 168 | 56 | 155 | 56 | 168 | 56 |
| 5 | 168 | 56 | 165 | 56 | 164.8 | 52 | 168 | 56 |
| 6 | 115 | 56 | 115 | 56 | 110 | 56 | 112 | 56 |
| 7 | 112 | 56 | 112 | 56 | 106 | 56 | 112 | 56 |
| 8 | 84 | 54 | 72 | 54 | 81.5 | 54 | 81.5 | 54 |
| 9 | 82.5 | 56 | 82.5 | 56 | 82.5 | 56 | 82.5 | 56 |
| 10 | 84 | 56 | 84 | 56 | 84 | 56 | 84 | 56 |
| 11 | 84 | 56 | 84 | 56 | 82.5 | 56 | 84 | 56 |
| 12 | 104 | 56 | 104 | 56 | 93.5 | 56 | 104 | 56 |
| 13 | 104 | 56 | 93 | 54 | 98 | 54 | 98 | 56 |
| 14 | Calved and ration changed | | 150 | 56 | 150 | 56 | 150 | 56 |
| 15 | | | 168 | 56 | 168 | 56 | 168 | 56 |
| 16 | | | 168 | 56 | 168 | 56 | 168 | 56 |
| 17 | | | 168 | 56 | 168 | 56 | 168 | 56 |
| 18 | | | 140 | 56 | 140 | 56 | 140 | 56 |
| Total... | 1,526 | 683 | 2,298.5 | 961 | 2,226.6 | 957 | 2,306.5 | 963 |

bone meal per day had very little effect on the rate of growth. Apparently the Jerseys that received the bone meal increased slightly faster in weight than those that did not receive bone meal. The two Holsteins showed exactly the reverse. There was a very close agreement in the height curves of the two groups.

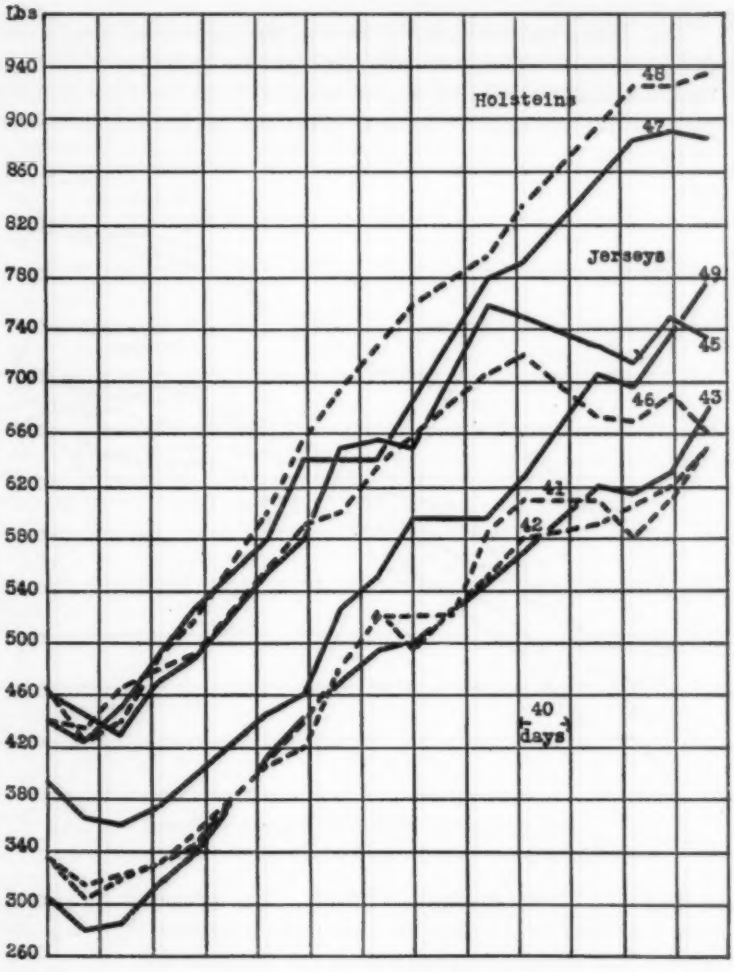


FIG. 1. GROWTH CURVES OF HEIFERS—WEIGHT
Solid lines indicate heifers that received 2 ounces bone meal daily

## DISCUSSION

The data presented can in no way be considered conclusive due to the small number of individuals used. It is shown however that the addition of 2 ounces of steamed bone meal per animal per day did not have a decided effect on the growth in weight

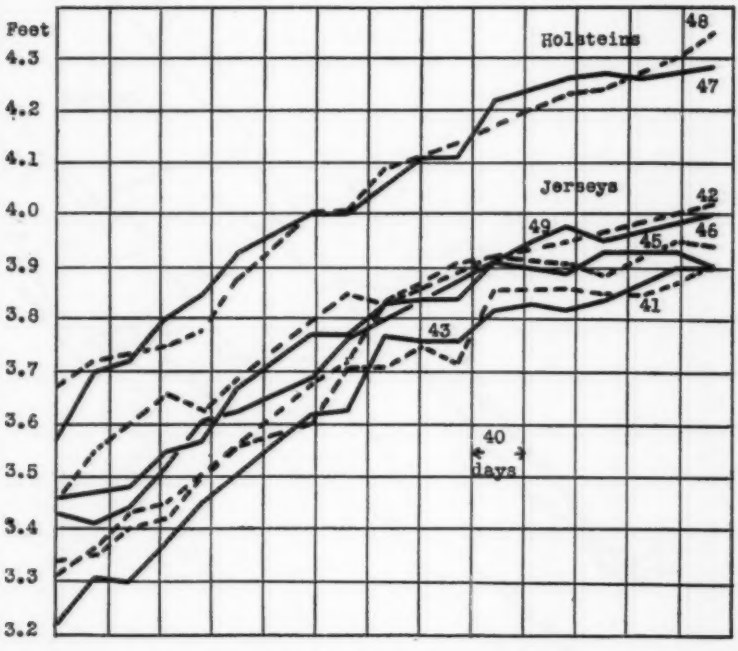


FIG. 2. GROWTH CURVES OF HEIFERS—HEIGHT
Solid lines indicate heifers that received 2 ounces bone meal daily

or height of 3 Jerseys and 1 Holstein. The character of growth was very similar to that of comparable heifers that did not receive any mineral supplement except common salt.

The ration used was rather low in calcium and the pasture was on land that has a low lime content. In view of these facts we expected the feeding of bone meal to be advantageous.

As the heifers calved they were placed in the milking[1] herd and the allowance of bone meal increased to 4 ounces daily. Birth weights and measurements of the calves were taken and the heifer calves will be continued on the same plan of mineral feeding as the dams. It is possible that the effect of mineral feeding will be reflected in the milk production or that it will show up in later generations.

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[1] Our milking herd consists of 3 groups: One group receives no mineral supplement except salt; one group receives salt and 4 ounces of bone meal per cow daily; and the other group receives salt and 4 ounces of $CaCO_3$ per cow daily.

# CARBONATION OF BUTTER*

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This study on the carbonation of butter is a part of a larger problem under investigation, namely, that of "The deterioration of dairy products and the means of prevention." As originally planned, the study included the use of other gases, particularly O, N and H. After some preliminary experiments all the other gases except carbon dioxide were given up because of the unfavorable results.

Carbon dioxide is used extensively and with success in the preparation of certain beverages (1, 2, 3, 4, 5, 6). Attempts have also been made to extend its use to the dairy products with the expectation that certain benefits would be derived therefrom. Van Slyke and Bosworth (7), published their study on the carbonation of milk in 1907. They showed that the charging of milk with the gas and keeping it under pressure delayed the souring of the milk. One of the authors, M. J. Prucha, who was then connected with the same institution, became interested in the subject after examining bacteriologically several samples of the carbonated milk. Since that time, the use of carbon dioxide for dairy products and other foods has been patented (8, 9). The dairy industry suffers heavy losses from the spoiling and the deterioration of the dairy products. Hence, any scheme, the aim of which is to improve these products and to prevent their spoilage, should receive careful consideration. A study on this subject was organized and has been carried on for more than three years. One report of this work, dealing with the carbonation of ice cream, has already been published (10). This paper presents the results from the experiments on the carbonation of butter.

Three different series of experiments were carried out. In the first series the carbon dioxide was applied to the sweet cream which was intended for butter. In the second series of experi-

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ments the carbon dioxide was applied at the time of churning, the butter being churned in the atmosphere of the gas. In the third series of experiments the butter was stored in carbon dioxide.

The main object of these experiments was to determine the effects of carbonation of cream and butter on the germ life, on the development of flavors, and on the keeping quality of these products.

The bacteriological work included both the counting of the bacteria, and the examination of the petri plates as to the kind of microbial flora that survived the carbonation. In some of the experiments yeast and mold counts were also made. Nutrient agar containing two per cent lactose was used for the bacteria, and acidified malted agar was used for the yeasts and the molds.

## CARBONATION OF SWEET CREAM

The experiments with the cream were conducted in a laboratory. Usually about 4 gallons of sweet cream were divided into four lots, two of which were carbonated and the other two were not. The cream was kept at two different temperatures, namely, at 40° and 70°F.

The carbonation was done as follows: A rubber hose was attached to the gas tank and to the loose end of the hose was attached a glass tube 2 feet long. The loose end of the glass tube was inserted into the cream as far as the bottom. The gas was then allowed to flow into the cream for three minutes at 5 pounds pressure. The cream was being stirred during the flow of the gas. The carbonation was repeated each day during the experiment.

At varying intervals of time samples of the cream were taken for the bacteriological examination and also for the acidity test. Two samples were always taken for the acidity test, one of which was heated to expel the carbon dioxide before applying the test.

The experiment was repeated at least ten times in addition to several preliminary trials. The results in all cases were so similar that only the results from one of the experiments are presented (table 1).

There are two points to be noticed in the data in table 1 as regards the influence of carbon dioxide on the bacteria in the cream. In the first place, the carbonation caused in most cases, but not always, a slight temporary reduction in the number of bacteria following the first application of the gas. The second point to be noticed is that the carbonation did not prevent the multiplication of the bacteria. Other experiments showed that

TABLE 1

*Effect of carbon dioxide on bacteria and on acidity in cream*

| AGE OF CREAM | CARBONATED | | | UNCARBONATED | | |
|---|---|---|---|---|---|---|
| | Number of bacteria per cubic centimeter | Acidity | | Number of bacteria per cubic centimeter | Acidity | |
| | | Before heating | After heating | | Before heating | After heating |
| Cream kept at room temperature | | | | | | |
| Fresh | 9,000,000 | 0.38 | 0.12 | 9,000,000 | 0.12 | 0.12 |
| 2 hours | 6,000,000 | | | 16,000,000 | | |
| 4 hours | 24,000,000 | | | 28,000,000 | | |
| 6 hours | 21,000,000 | | | 19,000,000 | | |
| 8 hours | 46,000,000 | | | 41,000,000 | | |
| 1 day | | 0.56 | 0.49 | | 0.49 | 0.44 |
| 2 days | 346,000,000 | 0.68 | 0.53 | 221,000,000 | 0.66 | 0.56 |
| 4 days | 245,000,000 | 0.81 | 0.51 | 307,000,000 | 0.60 | 0.49 |
| | | 0.65 | 0.45 | | 0.55 | 0.49 |
| Same cream as above but kept at 40°F. | | | | | | |
| Fresh | 9,000,000 | 0.28 | 0.12 | 9,000,000 | 0.12 | 0.12 |
| 3 hours | 11,000,000 | | | 17,000,000 | | |
| 6 hours | 12,000,000 | | | 18,000,000 | | |
| 8 hours | 8,000,000 | | | 23,000,000 | | |
| 1 day | | 0.28 | 0.14 | | 0.16 | 0.15 |
| 2 days | 35,000,000 | 0.50 | 0.16 | 55,000,000 | 0.25 | 0.24 |
| 4 days | 23,000,000 | 0.55 | 0.31 | 114,000,000 | 0.45 | 0.45 |
| 6 days | 103,000,000 | 0.7 | 0.41 | 312,000,000 | 0.50 | 0.43 |
| 7 days | | 0.7 | 0.42 | | 0.53 | 0.48 |

no amount of carbonation, unless under high pressure, will stop the bacteria from multiplying. After the temporary slight reduction the bacteria increased at about the same rate as they did in the uncarbonated cream.

From the examination of the petri plates prepared from the cream, it was very evident that the carbonated cream developed

somewhat different bacterial flora than the uncarbonated. Apparently the carbon dioxide checks certain types of bacteria and not other types. This probably explains the slight reduction due to carbonation.

The acidity test likewise showed that there was at first a slight retardation in the development of the acid in the carbonated cream; but, it also showed that this delay in the development of acidity was of short duration, and that both the carbonated and the uncarbonated cream soured, and that the degree of acidity in the end was about the same. It should also be mentioned that the uncarbonated cream became partially carbonated as it aged, due to the production of carbon dioxide through the activity of the germ life in the cream.

The effect of the carbonation on the keeping quality of the cream was slight and depended on the temperature at which the cream was held and probably on the amount and on the kind of initial bacterial contamination. At room temperature the carbonated cream soured in about the same length of time as the uncarbonated, a matter of two or three hours difference. At 40°F. the carbonated cream kept about two or three days longer.

From the commercial standpoint the carbonation of sweet cream for the purpose of improving the butter would not be practical. The difficulty of applying the carbon dioxide at the source of cream production, the expense involved, and the slight and doubtful benefits derived, would not make it economically profitable.

### CARBONATION OF BUTTER WHILE CHURNING

The experiments on the carbonation of butter while churning were conducted both in the laboratory and also in a commercial creamery. The carbon dioxide was applied in the following manner. In the laboratory experiments, a rubber hose was attached to the gas tank and the loose end of the hose was inserted into the cream as far as the bottom of the churn. The gas was then allowed to flow into the cream during the entire churning operation. In the creamery experiments, a four foot metal pipe was connected to the gas tank by means of a rubber hose. The

loose end of the pipe was inserted in the cream as far as the bottom of the churn and the gas was then blown in while the cream was being stirred until the cream was saturated and the gas escaped from the churn through the top opening. The churn was then closed and the churning operation was carried out as usual.

Approximately thirty experiments were done in the laboratory. In addition to the bacteriological study, the butter was stored at different temperatures and its keeping quality and the development of flavors were observed.

These laboratory experiments revealed no pronounced effect of the carbonation on the bacteria in the butter. The lactic acid bacteria, which constitute a large percentage of the bacteria in properly ripened cream, did not appear to be affected by the carbon dioxide.

The carbonated butter had a sourish taste immediately after the churning. This taste gradually disappeared in storage. In regard to the keeping quality of the butter, there was no pronounced difference between the carbonated and the uncarbonated butter except that the former tended to develop a slightly different off flavor. In these laboratory experiments it was impossible to find any benefits due to the carbonation that would be valuable enough to pay for the cost of the treatment.

The experiments in the creamery were done on a large scale and in the following manner. On three different days about 3000 gallons of cream were put in a holding tank after the cream was neutralized, pasteurized, and inoculated with a starter. The ripened cream was then drawn from this tank into the churns. The first three churnings, amounting to 2400 pounds of butter, were not treated. In the next three churnings the carbon dioxide was applied to the cream in the churns. The remaining cream was then carbonated in the holding tank. From each churning a sixty pound tub of butter was placed in cold storage at 40°F. from which to study the changes in the quality of the butter. The butter was scored by an expert judge at varying intervals of time. In one experiment the butter was held in the cold storage for 135 days and in the other two experiments only

TABLE 2

*Effect of carbonation on the keeping quality of butter*

| NUMBER OF CHURN | TREATMENT OF THE BUTTER | AGE OF THE BUTTER | | | | |
|---|---|---|---|---|---|---|
| | | 6 days | 20 days | 40 days | 90 days | 135 days |
| | | Butter score | | | | |
| | **First experiment** | | | | | |
| 1 | Not salted, not carbonated | 89 | 88 | 88 | 87.5 | 88.5 |
| 2 | Salted, not carbonated | 89.5 | 88.5 | 88.5 | 88.5 | 88.5 |
| 3 | Salted, not carbonated | 88.5 | 89 | 88 | 86.5 | 87 |
| 4 | Not salted, carbonated | 89.5 | 89 | 89 | 88 | 88 |
| 5 | Not salted, carbonated | 89.5 | 89 | 89 | 89 | 87.5 |
| 6 | Salted, carbonated | 88.5 | 89 | 88.5 | 87 | 87 |
| 7 | Salted, carbonated | 89 | 89.5 | 88 | 88 | 87.5 |
| 8 | Salted, carbonated | 89 | 90 | 88.5 | 88 | 88 |
| 9 | Salted, carbonated | 89.5 | 89.5 | 88.5 | 87 | 87.5 |
| 10 | Salted, carbonated | 89 | 87.5 | 86.5 | 86 | 87 |
| | **Second experiment** | | | | | |
| 1 | Not salted, not carbonated | 88.5 | 89.5 | 88 | | |
| 2 | Not salted, not carbonated | 90 | 88.5 | 87.5 | | |
| 3 | Not salted, not carbonated | 89 | 88 | 88 | | |
| 4 | Salted, carbonated | 88.5 | 88 | 88.5 | | |
| 5 | Salted, carbonated | 89.5 | 87 | 88.5 | | |
| 6 | Not salted, carbonated | 89.5 | 88 | 87.5 | | |
| 7 | Salted, carbonated | 90 | 88 | 88 | | |
| 8 | Salted, carbonated | 90 | 87 | 89 | | |
| 9 | Salted, carbonated | 90 | 88 | 88.5 | | |
| 10 | Salted, carbonated | 90.5 | 88 | 89 | | |
| 11 | Salted, carbonated | 87 | 86.5 | 84 | | |
| 12 | Salted, carbonated | 87 | 87 | 85 | | |
| | **Third experiment** | | | | | |
| 1 | Salted, not carbonated | 87 | 87 | 88 | | |
| 2 | Salted, not carbonated | 88 | 87 | 87.5 | | |
| 3 | Salted, not carbonated | 89 | 87.5 | 88.5 | | |
| 4 | Salted, carbonated | 89.5 | 88.5 | 87.5 | | |
| 5 | Salted, carbonated | 89.5 | 88.5 | 88.5 | | |
| 6 | Salted, carbonated | 90 | 88 | 88.5 | | |
| 7 | Salted, carbonated | 90 | 88.5 | 89 | | |
| 8 | Salted, carbonated | 88 | 88 | 88.5 | | |

*Note:* In churnings numbered 7 to 12 the cream was carbonated in the holding vat.

forty days. The results of the scoring of the butter are presented in table 2.

The numerical scores given to the butter by the judge as shown in table 2, do not reveal any measurable benefits nor any harm due to the carbonation. The carbonated butter would not have commanded a better price on the market than the uncarbonated. In some cases the carbonated butter was pronounced sourish. One lot of butter was made from cream which was slightly over

TABLE 3

*Number of bacteria, molds and yeasts in carbonated and uncarbonated cream and butter*

| CHURNING | NUMBER OF BACTERIA | | NUMBER OF YEASTS AND MOLDS | |
|---|---|---|---|---|
| | Per cubic centimeter cream | Per gram butter | Per cubic centimeter cream | Per gram butter |
| Not carbonated | | | | |
| 1 | 17,000,000 | 4,000,000 | 310 | 300 |
| 2 | 31,000,000 | 3,000,000 | 420 | 390 |
| 3 | 36,000,000 | 600,000 | 490 | 610 |
| Carbonated in churn | | | | |
| 4 | 33,000,000 | 2,000,000 | 310 | 1,400 |
| 5 | 32,000,000 | 4,000,000 | 480 | 240 |
| 6 | 32,000,000 | 2,000,000 | 390 | 240 |
| Cream carbonated in holding tank | | | | |
| 7 | 12,000,000 | 2,000,000 | 410 | 300 |
| 8 | 27,000,000 | | 290 | 400 |
| 9 | 25,000,000 | 3,000,000 | 230 | 800 |
| 10 | 14,000,000 | 4,000,000 | 5,600 | 800 |

neutralized and in this case the carbonation tended to cover this defect. The butter that was salted was free from molds while the unsalted butter became slightly moldy whether it was carbonated or uncarbonated.

Bacteriological counts were made from the cream immediately before churning and also from the butter when it was taken from the churn. The results from one day's run are given in table 3.

The bacteriological results show no significant effect of the carbonation. If any effect was produced it was not large enough to be detected by the bacteriological methods.

### STORING OF BUTTER IN CARBON DIOXIDE

In this series of experiments freshly made butter was placed in an atmosphere of carbon dioxide in an air tight vessel and then stored at different temperatures. At varying intervals of time the butter was examined for the development of flavors. This experiment was repeated about forty times. Bacteriological examination of the butter was also made in some of the experiments. The results from one of such experiments are given in table 4.

The bacterial counts in table 4 are very irregular. A partial explanation for this probably lies in the fact that the bacteria are unevenly distributed in the butter and that an accurate counting of bacteria in solid dairy products is difficult. But in spite of this variation the results indicate that the storing of butter in carbon dioxide tends to suppress the bacterial activity in the butter. At warmer temperature this suppression is of small duration and the bacteria soon increase in numbers. At colder temperature this suppression is more pronounced. The yeasts showed a slight increase which was largely controlled by the temperature.

There was one outstanding difference between the butter stored in carbon dioxide and that stored in the air, namely, that the butter stored in carbon dioxide was always free from the molds.

The keeping quality of the butter was also affected by the storage in the carbon dioxide. In most cases the butter stored in carbon dioxide kept a little longer before off flavors began to appear. This effect was also greatly influenced by the temperature and probably by some other causes. At a warm temperature the treated butter did not keep very much longer, while at a cold temperature it was a matter of several days and at times several weeks. However, this prolongation of the keeping quality of the butter was not permanent, for in time the treated butter also developed off flavors. The flavors in most cases were differ-

TABLE 4

*Bacterial, yeast and mold counts of the butter stored in carbon dioxide at different temperatures*

| AGE OF BUTTER | NUMBER OF BACTERIA PER GRAM | | NUMBER OF YEASTS AND MOLDS PER GRAM | |
|---|---|---|---|---|
| | Butter in $CO_2$ | Butter in air | Butter in $CO_2$ | Butter in air |
| **Butter kept at 70°F.** | | | | |
| 5 days —1/30/24 | 590,000 | 870,000 | 130,000 | 140,000 |
| 10 days —2/ 6/24 | 4,800,000 | 3,100,000 | 585,000 | 181,000 |
| 2 weeks—2/15/24 | 4,410,000 | 4,110,000 | 1,278,000 | 2,340,000 |
| 4 weeks—2/25/24 | 2,240,000 | 14,500,000 | 1,250,000 | 2,630,000 |
| 5 weeks—3/ 3/24 | 2,370,000 | 9,300,000 | 1,170,000 | 2,270,000 |
| 7 weeks—3/17/24 | 7,500,000 | 4,000,000 | 2,020,000 | 2,250,000 |
| **Butter kept at 60°F.** | | | | |
| 5 days —1/30/24 | 290,000 | 159,000 | 300 | 200 |
| 10 days —2/ 6/24 | 87,000 | 78,000 | 18,800 | 2,200 |
| 2 weeks—2/15/24 | 278,000 | 374,000 | 39,600 | 101,700 |
| 4 weeks—2/25/24 | 118,000 | 166,000 | 44,000 | 154,000 |
| 5 weeks—3/ 3/24 | 500,000 | 300,000 | | |
| 7 weeks—3/17/24 | | | 412,000 | 514,000 |
| 10 weeks—4/14/24 | 290,000 | 1,600,000 | 520,000 | 460,000* |
| **Butter kept at 50°F.** | | | | |
| 5 days —1/30/24 | 180,000 | 297,000 | 200 | 500 |
| 10 days —2/ 6/24 | 151,000 | 429,000 | 200 | 14,900 |
| 2 weeks—2/15/24 | 56,500 | 176,000 | 300 | 1,100 |
| 4 weeks—2/25/24 | 44,900 | 480,000 | 200 | 100,300 |
| 5 weeks—3/ 4/24 | 49,000 | 386,000 | 400 | 121,000 |
| 7 weeks—3/17/24 | 480,000 | 835,000 | 14,300 | |
| 10 weeks—4/14/24 | 108,000 | 1,600,000 | 290,000 | 229,000* |
| **Butter kept at 40°F.** | | | | |
| 5 days —1/30/24 | 445,000 | 250,000 | 200 | 300 |
| 10 days —2/ 6/24 | 68,000 | 40,000 | | 300 |
| 2 weeks—2/15/24 | 57,700 | 63,000 | | 200 |
| 4 weeks—2/25/24 | 582,000 | 98,600 | 800 | 24,200 |
| 5 weeks—3/ 4/24 | 110,000 | 90,000 | 300 | 50,000 |
| 7 weeks—3/17/24 | 440,000 | 730,000 | 800 | |
| 14 weeks—5/ 8/24 | 500,000 | 1,090,000 | 1,100 | 540,000 |
| **Butter kept at 32°F.** | | | | |
| 5 days 1/30/24 | 441,000 | 345,000 | 700 | 300 |
| 10 days 2/ 6/24 | 220,000 | 211,000 | 200 | |
| 4 weeks—2/26/24 | 176,000 | 178,000 | 250 | 450 |
| 7 weeks—3/17/24 | 83,000 | 156,000 | 650 | 5,900 |
| 14 weeks—5/ 8/24 | 299,000 | 1,400,000 | 48,000 | 98,000 |

* Many molds.

ent and occasionally even more objectionable than in the uncarbonated butter.

## SUMMARY AND CONCLUSIONS

The carbonation of sweet cream as done in these experiments tended to suppress certain types of bacteria but did not hinder other types of bacteria from their multipliction. This effect of the carbonation caused a delay in the souring of the cream. The length of time of this delay depended on the temperature at which the cream was held and probably also on the amount and on the kind of the initial bacterial contamination. At room temperature the delay was a matter of about two hours while at a temperature of 35°F. it was a matter of several days.

When the carbon dioxide was applied at churning time by charging the cream in the churn and by replacing the air above the cream in the churn by the gas, no significant benefit appeared to be derived therefrom. The gas caused the butter when fresh to taste sourish and this taste tended to cover the taste due to the neutralizer used in the cream. The molds were not inhibited by this treatment. Both the laboratory as well as the commercial creamery experiments showed that this method of application of carbon dioxide did not measurably affect the germ life in the butter nor did it cause the butter to have better flavors to to keep longer.

When the carbon dioxide was applied by storing the butter in the gas in an air tight container, it completely inhibited the development of the molds in the butter. It also prolonged the keeping quality of the butter when held at cold temperature. However, in time the butter stored in carbon dioxide also developed undesirable flavors.

The result of these experiments indicate that the carbonation of the cream intended for butter, or the carbonation of the butter during churning operation does not result in any special benefit to justify the use of carbon dioxide in this manner.

Hunziker (11), reported that the carbonation of butter as done commercially—that is, when applied at churning time—is of no special value to the butter industry.

Rettger, Winslow and Smith (12) found that the carbonation of ice cream as done commercially has no appreciable affect on the germ life or on the vitamin in the ice cream.

Prucha, Brannon and Ambrose (10) reported that they could find no significant effect of carbonation of ice cream on the germ life therein.

Such results are to be expected when the available information on the influence of carbon dioxide on germ life is carefully examined.

The carbonation of beverages is successful because the beverages are carbonated and are kept in air tight containers in this gas, under pressure. Donald Jones and Maclean recommended a pressure of 70 pounds. If these drinks were carbonated at the time they are prepared and then left in unsedled containers the beneficial effect of carbonation would disappear. Our results from experiments on milk kept under pressure indicate that much higher pressure is necessary in case of dairy products to completely inhibit the bacterial activities in them.

All the evidence from these studies point to the conclusion that in order to obtain any appreciable benefits from the carbonation of the butter, it is necessary to store the butter in the atmosphere of carbon dioxide in an air tight container. Whether the benefits derived from such treatment are of any economic value will have to be demonstrated commercially on a large scale.

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# SOME STUDIES IN REDUCING THE STABILITY OF BUTTERFAT EMULSIONS*

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The purpose of this study was to determine some of the factors which tend to stabilize emulsions of butterfat in buttermilk obtained by the churning of cream; and, if possible, to develop methods which would at least partially destroy the stability of the emulsion and render a more complete churning of the cream with a lower loss of fat in the buttermilk.

It is common knowledge that butterfat exists in cream in the form of minute globules, held in more or less stable "oil-in-water" emulsion. The churning of cream consists primarily in subjecting it to violent agitation, so that the globules of fat are forced in contact with each other. If this is done at the proper temperature the globules will break through the thin film of milk serum, separating them from one another and coalesce, until finally these adhering globules form butter granules which are large enough to be readily separated from the fluid buttermilk in which they float. In other words, the mechanical churning breaks down the oil-in-water emulsion and forms butter, which is in reality a water in oil emulsion. This change, however, is not complete, and a certain amount of fat remains in a rather stable oil in water emulsion, which constitutes a fat loss to the butter manufacturer.

There are numerous factors affecting the losses of fat in buttermilk and many of these, such as correct temperature, fullness of the churn, and acidity of the cream are carefully watched and controlled in order to keep the fat losses down to a minimum. Nevertheless, there is a considerable loss. The average fat content of fifty-seven samples of buttermilk secured from various

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creameries in Illinois, was 0.914 per cent. Under existing conditions this fat which is lost seems to be in such a stable emulsion as to be unchurnable.

There are, no doubt, several factors responsible for this extreme stability. The components of milk or cream existing normally in colloidal solution act as stabilizing agents. Much of the cream received in butter manufacturing plants has a high acid content, and in order to properly prepare this cream for pasteurization, a portion of the acid is reduced by the addition of solutions of various kinds of alkali. Those most commonly used are calcium lime, calcium-magnesium lime, sodium carbonate, and sodium bicarbonate. There is no doubt that this neutralizing process is not always carried out properly and that at times, a certain amount of saponification takes place. Donnon (1) has shown by his work that soap is an excellent emulsifying agent for fat. Other investigators (2) have also verified the results obtained by Donnon.

There are also certain compounds formed by the action of these alkalies upon the proteins of the cream and these products are excellent emulsifying agents, and no doubt, are important factors in stabilizing the emulsion of fat in buttermilk. The influence of electrical charges in stabilizing colloidol emulsions, may also be a factor.

Hardy (3) has suggested that when colloidal partiles are coagulated under the influence of electrolytes, the fat globules absorb oppositely charged ions, so that their charges become neutralized, and at the iso-electric point, stability is lost (2). Ellis (4) found that the rate of coagulation of the oil globules was practically independent of the interfacial tension, depending only on the electrical potential at the surface of the globules. On adding HCl to an oil-in-water emulsion, the interfacial tension did not vary within the limits of experimental error; while the stability decreased enormously as the acid was added (2).

The conclusions of Ellis seem somewhat in disagreement with the work of Bredig (5), who reports that there is a decrease of surface tension with coagulation. However, Ellis considers the electrical factors of far more importance than the effect of the interfacial tension.

Early investigators considered viscosity as an important factor in stabilizing oil-in-water emulsions. At the present time, however, many investigators in this field do not consider viscosity as a cause for emulsification, but rather that viscosity aids emulsification solely by virture of the of the hindrance offered to the coalescence of the dispersed fat globules.

According to Clayton (2), the hydration theory of emulsions as proposed by Fisher (6) is subject to several objections. This hydration theory considers it necessary to have a certain minimum amount of water present in order to emulsify oil in a hydrated colloid such as casein. Furthermore, it is considered that if water is present in an amount greater than that used in hydrating the colloid, no stable emulsion is possible.

This theory does not seem applicable to the problem under consideration, since in buttermilk water is present in an amount very much in excess of that necessary to hydrate the casein; and yet, there is a stable emulsion of fat in buttermilk.

In addition to the factors pointed out above, there is also a possibility that considerable fat is lost in buttermilk, due either to mechanical entrapping of some of the minute globules of fat in casein particles, or by the adsorption of fat globules by these components of the buttermilk.

The relative importance of this theory as compared with the other theories mentioned above could readily be ascertained by separating buttermilk into its two component parts—curd and whey, and determining the fat content of each. This procedure was followed. Special attention, however, was given to investigating the electrolyte theory of stability of fat emulsions, the substances used as electrolytes, were HCl, NaCl, Th $(NO_3)_4$, oleic acid and other chemicals. Since butter is a food, nothing could be used that would impair its wholesomeness, nor destroy the possibilities of the buttermilk being used for feeding purposes.

## METHOD OF PROCEDURE

To determine the proportion of whey and curd in a sample of buttermilk, the following method was used: A heavy glass tube was filled with a known weight of well mixed buttermilk and

centrifuged for seventy-five minutes at a speed of 1200 r.p.m. (a pressure of 40.6 pounds per square inch was generated). This force was sufficient to cause a complete separation of the curd and whey into distinct layers, which could be separated and analyzed. The amount of fat in the curd and whey was determined by multiplying their weights and tests, respectively, and from this the relative amounts of fats were calculated, using the total in the buttermilk as 100 per cent.

The fat tests on all samples were determined by using the Mojonnier tester. For buttermilk and whey, a 10-gram sample was measured into an extraction flask, 1.5 cc. of ammonia water was added and the flask shaken. Sufficient alcohol was added to bring the contents up to the top of the small neck of the bulb and the flask again shaken. Twenty-five cubic centimeters of petroleum ether were added and the total contents thoroughly mixed. The flask was centrifuged in order to separate the ether solution of fat, and this was decanted into a weighed dish. This extraction was repeated using 15 cc. of ethyl and petroleum ether, respectively. The second extraction was added to the dish containing the first, and the solution evaporated to dryness, being heated to a constant weight at 135°F. under a 25-inch vacuum. The dishes were cooled, weighed, and the percentage of fat calculated.

The fat in the curd was determined as follows: 2 to 3 grams of curd were weighed into a Mojonnier butter boat, and then placed into an extraction flask. Ten cubic centimeters of hot water were added, and after the contents were mixed thoroughly, the procedure was carried out as described for buttermilk and whey.

Forty-nine samples of buttermilk were tested to determine the proportion of curd and whey and their fat contents. These samples were secured from cream which had been neutralized with the following substances: rock lime, Mitchell lime, Alwood lime, soda ash, a combination of Alwood lime and soda ash, and an unneutralized check sample. The strength of each of the neutralizers was approximately 15 per cent, except in the case of soda ash when a 10 per cent solution was used. Samples of buttermilk were taken from the same churn at the start and near the

end of the draining, in order to determine if there was any variation in the fat content.

Working on the theory that hydrochloric acid or sodium chloride would neutralize the electrical charge and break the emulsion of the fat, churnings were made in the following ways: A large batch of cream was neutralized, pasteurized and cooled to churning temperature. It was divided into three lots of equal size and churned as nearly the same time as possible, the only variation in each churning being the treatment given the cream in the churn. Lot 1 was churned with no treatment and was used as the check churning. One per cent salt was added to lot 2 and to lot 3, sufficient hydrochloric acid was added to increase the acidity 0.05 per cent, calculated as lactic acid. The acid and salt were added to the cream as it went into the churn.

Churnings were repeated in exactly the same manner except that a combination of 1 per cent salt and sufficient acid to increase the acidity of the cream 0.05 per cent, calculated as lactic acid, were used.

Later, the salt and acid method was carried out on a commercial scale. The cream was prepared by being neutralized with Mitchell lime and pasteurized, and immediately before churning, the batch was divided into two equal parts of 1900 pounds. These lots were churned simultaneously in 1000-pound Dual churns, driven by the same shaft and motor. One churning was untreated and was used as a check. To the other, 490 cc. of 37 per cent hydrochloric acid and 19 pounds of salt were added. The time to churn, temperature, etc., were recorded on each.

The work was repeated in another creamery where Alwood lime was used as a neutralizer for the cream, and where three churnings of 2600 pounds were secured from the same vat of cream. One churning was used as the check, to the second, 25 cc. of hydrochloric acid and 1 pound of salt were added for each 100 pounds of cream and the third churning was treated with varying amounts of acid and salt to determine the effect of varying concentrations of these substances.

Another series of thirty-three experiments were repeated in the second plant, substituting Peerless hydrated lime for Alwood

lime. The method of procedure was exactly the same as in the previous experiments except in this set of experiments, two pails of cold water were added to some of the churnings when the butter began to break to determine the effect upon the emulsified fat.

Acidity tests were made upon the butter in order to determine the effect of the HCl upon the acidity of the finished product. The following method, described by Bouska (8), was used. Nine grams of butter were melted to the consistency of thick cream. To this were added 20 cc. of a mixture of 100 parts ether and 25 parts of alcohol. This was titrated with N/20 NaOH, using phenolphthalein as an indicator and the acidity computed.

In some churnings, other chemicals were used in the same way as the HCl and NaCl in an attempt to destroy the emulsion. Di-sodium phosphate, sodium citrate, 10 per cent solution of oleic acid and thorium nitrate were added to cream for this purpose.

## DISCUSSION OF RESULTS

The largest part of all buttermilk samples was whey, as was shown by separating the curd and the whey by centrifugal force. Table 1 shows the maximum percentage to be 90 per cent; the minimum, 79.7 per cent; and the average of 79 samples, 82.5 per cent.

There is no set rule as to where the fat in buttermilk is found. The greatest portion may be held in the curd or in the whey, depending upon whether or not the cream is neutralized and upon the neutralizer used, and also upon other conditions affecting losses in churning, such as acidity, temperature, speed of churn, time held, etc. Buttermilk from cream, which was neutralized with soda ash, contained approximately three-fourths of the fat in the whey, whereas, that from unneutralized cream, contained three-fourths of the fat in the curd. The other neuralizers varied between these two extremes. The average of all buttermilk samples was 47.8 per cent of the fat in the whey and 52.2 per cent in the curd.

The analyses of samples of buttermilk taken at the start and at

the end of draining showed no variation in the fat content. In ten experiments, the maximum variation between two samples of buttermilk from the same churning was 0.018 per cent; the minimum, 0.004 per cent; and the average, 0.009 per cent. A buttermilk sample taken at any time during draining will be representative providing no fat granules get into the buttermilk. Probably,

TABLE 1

*The relative amount of whey contained in buttermilk obtained from cream untreated and treated with HCl and NaCl*

| TREATMENT | NUMBER OF SAMPLES | MAXIMUM WHEY | MINIMUM WHEY | AVERAGE WHEY |
|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* |
| None | 49 | 88.9 | 80.9 | 82.3 |
| Salt | 11 | 90.0 | 79.7 | 85.5 |
| HCl | 11 | 86.0 | 69.6 | 80.3 |
| HCl and salt | 8 | 85.7 | 77.5 | 81.9 |
| Total or average | 79 | 87.6 | 78.9 | 82.5 |

TABLE 2

*The relative amount of fat contained in the whey and curd of buttermilk from cream unneutralized and neutralized*

| NEUTRALIZER | FAT IN WHEY | | | FAT IN CURD | | |
|---|---|---|---|---|---|---|
| | Maximum | Minimum | Average | Maximum | Minimum | Average |
| Rock lime (CaO) | 68.1 | 30.3 | 54.2 | 69.7 | 31.9 | 45.8 |
| Mitchell lime | 84.0 | 32.4 | 46.1 | 67.6 | 16.0 | 53.9 |
| Alwood lime (Mg. lime) | 63.4 | 38.3 | 48.3 | 61.7 | 36.6 | 51.7 |
| Soda ash | 84.4 | 51.0 | 71.8 | 49.0 | 15.6 | 28.2 |
| Alwood lime and soda ash | 59.8 | 22.3 | 26.3 | 77.7 | 40.2 | 58.8 |
| None | 27.6 | 25.1 | 26.3 | 74.9 | 72.4 | 73.7 |
| Average of all | 64.5 | 33.2 | 47.8 | 66.8 | 35.5 | 52.2 |

the best time to take a sample is just after the first buttermilk has passed off (which washes away any cream from the churn gate) and while the butter granules are still floating in the churn.

Table 3 shows the results of churning different lots of cream, each divided into three parts and treated as indicated. Both salt and acid when used separately had some effects in liberating the fat in the curd and whey.

The results tend to show that churning with acid sets free a larger amount of fat from the curd than from the whey, since a larger percentage of the total fat was found in the whey when acid was used, than in the check churning. The tendency of the salt was to free more from the curd than did the acid. The

TABLE 3

*Use of NaCl and HCl in churning*

| NEUTRALIZER | TREATMENT | BUTTERMILK TEST | RELATIVE AMOUNT OF FAT | | AVERAGE REDUCTION IN TEST OF BUTTERMILK DUE TO TREATMENT |
|---|---|---|---|---|---|
| | | | In whey | In curd | |
| Lime | None | 1.048 | 54.1 | 45.9 | |
| | Salt | 0.858 | 77.4 | 32.6 | 0.190 |
| | HCl | 0.720 | 70.7 | 39.3 | 0.328 |
| Soda ash | None | 0.782 | 71.8 | 28.2 | |
| | Salt | 0.563 | 77.5 | 22.5 | 0.219 |
| | HCl | 0.484 | 75.0 | 25.0 | 0.298 |
| Alwood lime | None | 0.832 | 48.3 | 51.7 | |
| | Salt | 0.636 | 63.9 | 36.1 | 0.196 |
| | HCl | 0.602 | 59.5 | 40.5 | 0.230 |
| Mitchell lime | None | 1.709 | 46.2 | 53.8 | |
| | Salt | 1.162 | 71.9 | 28.1 | 0.547 |
| | HCl | 0.882 | 59.2 | 40.8 | 0.827 |
| Alwood lime and soda ash | None | 0.849 | 41.2 | 58.8 | |
| | Salt | 0.667 | 57.7 | 42.3 | 0.182 |
| | HCl | 0.503 | 49.0 | 51.0 | 0.346 |
| All neutralizer average | None | 1.044 | 52.3 | 47.7 | |
| | Salt | 0.777 | 65.7 | 34.3 | 0.267 |
| | HCl | 0.638 | 62.7 | 37.3 | 0.406 |

results showed a greater amount of the fat was held in the whey with salt than with acid or the check churning.

Table 4 shows the results obtained when a combination of acid and salt were used. The addition of this combination tends to free the fat held in the curd more than does either when used

separately. It also tends to liberate more fat from the curd than it does from the whey. In each case, the larger percentage of fat was found in the whey than in the check or untreated sample.

The average reduction of fat losses in buttermilk, when salt was added, was 0.267 per cent as compared with the check churning and it was 0.406 per cent when acid was used. When a combination of both was used, the reduction was 0.480 per cent.

TABLE 4

*Results of churnings to which both HCl and salt were added*

| NEUTRALIZER | TREATMENT | BUTTERMILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| Alwood lime | None | 1.010 | 53.2 | 46.8 | |
| | Salt and acid | 0.456 | 86.0 | 14.0 | 0.554 |
| Mitchell lime | None | 1.221 | 47.8 | 52.2 | |
| | Salt and acid | 0.704 | 79.6 | 30.4 | 0.517 |
| Alwood lime and soda ash | None | 0.721 | 48.3 | 51.7 | |
| | Salt and acid | 0.352 | 69.0 | 31.0 | 0.369 |
| All neutralizers averaged | None | 0.984 | 49.8 | 50.2 | |
| | Salt and acid | 0.504 | 78.2 | 21.8 | 0.480 |

TABLE 5

*Results obtained by the use of salt and HCl in commercial plant where Mitchell lime was used for neutralizing the cream*

| TREATMENT | | BUTTERMILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| None | Maximum | 1.012 | 32.4 | 67.6 | |
| | Minimum | 0.644 | 46.1 | 53.9 | |
| | Average | 0.828 | 39.2 | 60.8 | |
| Salt and HCl | Maximum | 0.720 | 60.0 | 40.0 | 0.292 |
| | Minimum | 0.232 | 82.3 | 17.7 | 0.412 |
| | Average | 0.476 | 71.1 | 28.9 | 0.352 |

Tables 5 to 7 give the results obtained in commercial plants. In the first two plants, acid and salt were used. One per cent of salt was added to the cream and sufficient 37 per cent hydrochloric

acid to increase the acidity of the cream 0.05 per cent, calculated as lactic acid. Table 7 gives the results obtained on a second

TABLE 6

*Results obtained by the use of NaCl and HCl in commercial plant where Alwood lime was used for neutralizing*

| TREATMENT | | BUTTER-MILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| None | Maximum | 0.758 | 48.0 | 52.0 | |
| | Minimum | 0.422 | 23.9 | 76.1 | |
| | Average | 0.598 | 34.1 | 65.9 | |
| Salt and HCl | Maximum | 0.540 | 85.0 | 15.0 | 0.218 |
| | Minimum | 0.282 | 31.9 | 63.1 | 0.140 |
| | Average | 0.443 | 55.2 | 44.8 | 0.155 |

TABLE 7

*Results obtained by the use of salt and HCl in a commercial plant where Peerless hydrated lime was used*

| TREATMENT | | BUTTER-MILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| | | | *per cent* | *per cent* | |
| None | Maximum | 1.014 | 30.2 | 69.8 | |
| | Minimum | 0.706 | 19.0 | 81.0 | |
| | Average | 0.780 | 24.4 | 75.6 | |
| Salt and acid | Maximum | 0.762 | 34.5 | 65.5 | 0.252 |
| | Minimum | 0.522 | 20.2 | 79.8 | 0.144 |
| | Average | 0.636 | 27.6 | 72.4 | 0.184 |
| $H^2O$ added as cream breaks* | Maximum | 0.694 | 25.8 | 74.2 | 0.178 |
| | Minimum | 0.660 | 21.0 | 79.0 | 0.046 |
| | Average | 0.677 | 23.4 | 76.6 | 0.080 |
| Water, acid and salt* | Maximum | 0.734 | 31.2 | 68.8 | 0.280 |
| | Minimum | 0.562 | 26.2 | 73.8 | 0.144 |
| | Average | 0.621 | 28.8 | 71.2 | 0.159 |

* Two 12-quart pails of cold water were added to the churn when the cream started to break.

visit to the plant in which the results of table 6 were secured. The only variation was the substitution of Peerless hydrated lime for Alwood Lime.

separately. It also tends to liberate more fat from the curd than it does from the whey. In each case, the larger percentage of fat was found in the whey than in the check or untreated sample.

The average reduction of fat losses in buttermilk, when salt was added, was 0.267 per cent as compared with the check churning and it was 0.406 per cent when acid was used. When a combination of both was used, the reduction was 0.480 per cent.

TABLE 4

*Results of churnings to which both HCl and salt were added*

| NEUTRALIZER | TREATMENT | BUTTERMILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| Alwood lime | None | 1.010 | 53.2 | 46.8 | |
| | Salt and acid | 0.456 | 86.0 | 14.0 | 0.554 |
| Mitchell lime | None | 1.221 | 47.8 | 52.2 | |
| | Salt and acid | 0.704 | 79.6 | 30.4 | 0.517 |
| Alwood lime and soda ash | None | 0.721 | 48.3 | 51.7 | |
| | Salt and acid | 0.352 | 69.0 | 31.0 | 0.369 |
| All neutralizers averaged | None | 0.984 | 49.8 | 50.2 | |
| | Salt and acid | 0.504 | 78.2 | 21.8 | 0.480 |

TABLE 5

*Results obtained by the use of salt and HCl in commercial plant where Mitchell lime was used for neutralizing the cream*

| TREATMENT | | BUTTERMILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| None | Maximum | 1.012 | 32.4 | 67.6 | |
| | Minimum | 0.644 | 46.1 | 53.9 | |
| | Average | 0.828 | 39.2 | 60.8 | |
| Salt and HCl | Maximum | 0.720 | 60.0 | 40.0 | 0.292 |
| | Minimum | 0.232 | 82.3 | 17.7 | 0.412 |
| | Average | 0.476 | 71.1 | 28.9 | 0.352 |

Tables 5 to 7 give the results obtained in commercial plants. In the first two plants, acid and salt were used. One per cent of salt was added to the cream and sufficient 37 per cent hydrochloric

acid to increase the acidity of the cream 0.05 per cent, calculated as lactic acid. Table 7 gives the results obtained on a second

TABLE 6

*Results obtained by the use of NaCl and HCl in commercial plant where Alwood lime was used for neutralizing*

| TREATMENT | | BUTTERMILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| None | Maximum | 0.758 | 48.0 | 52.0 | |
| | Minimum | 0.422 | 23.9 | 76.1 | |
| | Average | 0.598 | 34.1 | 65.9 | |
| Salt and HCl | Maximum | 0.540 | 85.0 | 15.0 | 0.218 |
| | Minimum | 0.282 | 31.9 | 63.1 | 0.140 |
| | Average | 0.443 | 55.2 | 44.8 | 0.155 |

TABLE 7

*Results obtained by the use of salt and HCl in a commercial plant where Peerless hydrated lime was used*

| TREATMENT | | BUTTERMILK TEST | FAT | | FAT REDUCTION |
|---|---|---|---|---|---|
| | | | Whey | Curd | |
| | | | *per cent* | *per cent* | |
| None | Maximum | 1.014 | 30.2 | 69.8 | |
| | Minimum | 0.706 | 19.0 | 81.0 | |
| | Average | 0.780 | 24.4 | 75.6 | |
| Salt and acid | Maximum | 0.762 | 34.5 | 65.5 | 0.252 |
| | Minimum | 0.522 | 20.2 | 79.8 | 0.144 |
| | Average | 0.636 | 27.6 | 72.4 | 0.184 |
| $H^2O$ added as cream breaks* | Maximum | 0.694 | 25.8 | 74.2 | 0.178 |
| | Minimum | 0.660 | 21.0 | 79.0 | 0.046 |
| | Average | 0.677 | 23.4 | 76.6 | 0.080 |
| Water, acid and salt* | Maximum | 0.734 | 31.2 | 68.8 | 0.280 |
| | Minimum | 0.562 | 26.2 | 73.8 | 0.144 |
| | Average | 0.621 | 28.8 | 71.2 | 0.159 |

* Two 12-quart pails of cold water were added to the churn when the cream started to break.

visit to the plant in which the results of table 6 were secured. The only variation was the substitution of Peerless hydrated lime for Alwood Lime.

The results obtained in the commercial plants were very similar to those obtained in preliminary work carried on in the laboratory. The fat content of the curd was lowered by the acid and salt. The results obtained when Peerless lime was used, were somewhat different from those obtained with the other lime. When the results in table 7 are compared to those in tables 5 and 6, it will be noticed that fat reductions were secured in each, however, there was an outstanding difference in the fat content of the curd in the treated as compared with the check churning. Neutralizers seem to have a tendency to cause such variations. When Peerless lime was used, the fat reduction came from both the curd and whey in practically the same proportion. On the other hand, when either Mitchell or Alwood lime was used, the reduction occurred more in the curd than the whey. The effect that the various neutralizers have in forming a compound which stabilizes the emulsion is another problem yet to be solved.

The adding of water to the churn as the cream started to break did not lower materially the per cent of fat in the buttermilk. Some special emulsions can be destroyed by dilution, however, our results do not show this to be true with buttermilk. The reduction secured was due to dilution, since the adding of two buckets of water increased the volume of buttermilk. The combination of water, salt and acid did not decrease the fat loss any more than did the salt and acid, except that caused by dilution of the buttermilk.

By comparing table 5 with tables 6 and 7, it can be seen that in the one plant where a more exhaustive churning was secured with no treatment, the average reduction was less when salt and acid were used than in the plant with the larger fat losses. The first plant has an average fat loss of 0.828 per cent and the second 0.689 per cent. With the use of acid and salt, the percentage fat in the buttermilk was reduced to 0.476 per cent in the first plant, and 0.539 per cent in the second. The minimum for each plant was nearly the same. This tends to show that there was a certain amount of emulsified fat in the whey, and some held by the curd, which was not churnable under the conditions used.

The other chemicals, which were used, did not give results

which were at all promising. The average reduction in the test of the buttermilk secured with di-sodium phosphate was 0.06 per cent. Results obtained with sodium citrate showed only an average reduction of 0.02 per cent. These results come within the limits of experimental error.

In one set of experiments, 5 cc. of a 10 per cent solution of oleic acid was added to 2 liters of cream in addition to the salt and HCl. These were compared with untreated churnings. This treatment reduced the fat in the buttermilk to 0.48 per cent.

TABLE 8

*Use of thorium nitrate*

| LOT NUMBER | TREATMENT | AMOUNT OF THORIUM NITRATE USED | THORIUM NITRATE IN LOT OF CREAM | REDUCTION OR INCREASE DUE TO THORIUM NITRATE |
|---|---|---|---|---|
| | | *grams* | *per cent* | |
| | Unneutralized | 4.62 | 0.019 | −0.13 |
| 2 | Lime used | 9.46 | 0.044 | +0.71 |
| 3 | Lime used | 4.62 | 0.018 | +0.14 |

TABLE 9

*Effect of the use of HCl on the acidity of the butter*

| LOT NUMBER | TREATMENT | ACIDITY OF BUTTER |
|---|---|---|
| 1 | None | 0.1405 |
| | HCl | 0.1385 |
| 2 | None | 0.1525 |
| | HCl | 0.1550 |

Further trials with this treatment were not attempted because the finished product had a decided off flavor of oleic acid which would eliminate its use.

Clayton (2), states that thorium nitrate will invert an emulsion. A series of experiments were made using this material. The results obtained are given in table 8.

The results obtained with thorium nitrate do not indicate that it would prove of value in destroying the fat emulsion in buttermilk.

The use of HCl did not seem to have any effect upon the total

acidity of the butter, as is shown in table 9. Apparently the HCl is removed in the buttermilk and wash water.

The 1 per cent of salt used is largely removed with the buttermilk and wash water, although a small amount is retained by the butter. Analyses of the butter showed that it contained from 0.2 to 0.3 per cent salt, the average being 0.25 per cent.

The quality of the butter as determined by three judges, was not affected by the salt and acid treatment. One of the judges was consistent in scoring the treated samples about one-half point higher than the untreated butter. This was possibly due to the fact that the treated sample did contain a small amount of salt, whereas the check sample was unsalted.

## CONCLUSIONS

1. Seventy-nine samples of buttermilk which had been subjected to centrifugal force and separated into the components, whey and curd, gave an average whey content of 82.5 per cent.

2. The fat contained in buttermilk is partly held in stable emulsion in the whey, and partly in the curd, either by adsorption or mechanical entrapping. The relative amounts held by the curd and whey are variable and dependent upon numerous factors such as the kind of neutralizer used, acidity and temperature of the cream, speed of churn, etc.

3. The addition of 1 per cent of NaCl to the cream before churning will tend to decrease fat losses in the buttermilk. A small amount of salt, 0.2 to 0.3 per cent, is incorporated in the butter.

4. The addition of HCl to the cream before churning is more affective than NaCl in reducing the fat loss in buttermilk. The HCl added to the cream apparently does not increase the total acidity of the butter.

5. The use of the combination of NaCl and HCl is more affective in reducing fat losses in churning than either of these when used alone.

In this investigation it was not possible to reduce the fat content of buttermilk from neutralized cream, below an average of approximately 0.5 per cent.

6. The use of di-sodium phosphate, sodium citrate, or thorium nitrate did not prove effective in decreasing the fat content of buttermilk. Oleic acid, because of its deteriorating effect on the quality of the butter, was eliminated as being an unsatisfactory substance to use.

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# A STUDY OF THE ORGANISMS CAUSING THICKENING OF SWEETENED CONDENSED MILK*

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Sweetened condensed milk, as found in the market, usually flows freely when removed from the can. The contents have a color closely resembling that of cream, and possess a pleasing odor and taste. Occasionally, however, one finds a can with the contents so thick that they will not flow without vigorous stirring, or, perhaps, are even solid.

Two different kinds of thickening that may cause this condition have been observed. One of these, commonly called heat or age thickening, apparently causes a change only in the viscosity of the milk, the taste and odor remaining normal. The other kind of thickening, which is considered in this paper, produces different conditions. In the early stages, the milk may show a normal body having clots of thickened milk throughout. Sometimes the milk may be so thick that it will barely flow. In later stages it may be solid, so that it is possible to invert the can without the loss of its contents. As the degree of thickening increases, acidity and a characteristic fruity odor develops, as well as a disagreeable taste. When vigorously stirred, the milk thins slightly, but it returns to its original thickness after standing. When a portion of the milk is diluted with water and heated, a separation of the curd takes place. By this test it can be distinguished from heat or age thickened milk.

This kind of thickening commonly referred to as bacterial thickening has been found to occur most commonly in late spring or summer or in shipments to tropical countries.

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## LITERATURE

Probably the first to report such a spoilage was Greig-Smith (1) who described a type of thickening in which he found large numbers of coccus forms of bacteria. He isolated a form with which he was able to again produce thickening in condensed milk. The organism he described as being a coccus was distinguished from Micrococcus pyogenes albus only by its being non-pathogenic to mice. The thermal death point in milk was found to be 63°C. for ten minutes.

From the fact that he was able to isolate the organism from samples of condensed milk manufactured in Switzerland and Norway that did not show signs of thickening in any way, he concluded that the organism was of widespread occurrence.

Hunziker (2) discusses "thickened and cheesy condensed milk" also, and points out that the conditions can be prevented by the use of large amounts of sugar in manufacture.

Rice and Downs (3) also report this type of thickening from the manufacturer's point of view, especially in regard to standardization and temperature influences.

## EXPERIMENTAL

It was the purpose of this work to study the organisms causing bacterial thickening, to determine whether or not they were common in condensed milk as found on the market, and if possible to determine their distribution.

In carrying out the study, organisms were isolated from a sample of thickened condensed milk which had been returned by the trade and from factory contamination. A large number of organisms were also isolated from samples of condensed, powdered and market milk as well as from the air. These, together with a group of coccus forms obtained from several collections of type cultures, were studied. Organisms isolated from thirty-six samples of fluid milk and others isolated from eleven samples of commercial condensed milk were also studied.

The organisms obtained were tested for their thickening ability in sweetened condensed milk by the procedure given below.

Those giving positive results were studied further to determine their cultural and biochemical characteristics so that they might be classified.

*Procedure used in the thickening tests*

Each lot of sweetened condensed milk used for the thickening tests was analyzed and standardized with sterile water to 71 per cent total solids. Lots 1, 2 and 3 were then put into sterile quart milk bottles, pasteurized at 145° to 150°F. for thirty minutes and cooled to 50°F. All other lots of milk used were pasteurized in a shot-gun can at a temperature of 150° to 155°F. for one hour. This change was made because the checks on lot 3 showed that some of the thickening organisms had survived the treatment given it.

After being cooled, 170 grams of each sample of milk was put into sterile glass-stoppered bottles.

When the organisms to be tested had been invigorated in 1 per cent sucrose broth for several days, 5 cc. of the culture was added to a bottle of pasteurized condensed milk. It was mixed well by vigorous shaking, and was then incubated at 30°C. for six weeks. With the 5 cc. of broth added, the total solids of the test milk was approximately 70 per cent.

Check bottles of each lot of milk were kept, and in every instance, with the exception of lot 3, the pasteurized milk gave negative results. Check bottles of 70 and 71 per cent unpasteurized milk all thickened except those in lot 2. This would seem to indicate that the thickening organism is often present in commercial condensed milk, and that when the solids are lowered thickening will take place. The lots of milk just described were used in all thickening tests as carried out with cultures of each series except where otherwise stated.

ISOLATION OF THE THICKENING ORGANISM

*a. From thickened samples of commercial milk*

The first attempt to isolate the thickening organism was made on commercial sweetened condensed milk which had thickened

and had developed the fruity odor and the disagreeable taste already mentioned. The acidity shown by titration was found to be high, and bacterial examination showed many thousands of coccus forms to the gram. Further study showed that the milk contained a practically pure culture of these cocci. The colonies appeared to be of two kinds, one white and the other straw color. On agar slants, they produced a filiform, white-to-transparent growth. When they were inoculated into litmus milk, it became acidified and a curd formed which contracted and slowly peptonized, leaving a pink, acid whey.

These cultures were inoculated into sweetened condensed milk containing approximately 70 per cent total solids. After the milk had been incubated at 30°C. for a few weeks, the samples thickened and developed the characteristic fruity odor and high acidity. It was found that this thickened milk, when mixed with water, went into solution very slowly. When a portion of the diluted milk was heated, coagulation took place and the curd separated.

From this data it seemed apparent that the organism isolated was similar to the one described by Greig-Smith. It was evident that the high titratable acidity which accompanied the thickening and the acid peptonization of the litmus milk tubes would be valuable determinative tests which could be used in the study.

### *b. From factory contamination*

As no other samples of thickened milk were available for this study, an experiment was planned with the hope that a thickened sample of milk could be produced. It was quite evident that if the thermal death point of the organism was 63°C., as reported by Greig-Smith, none of the thickening organisms would survive the condensing process. Therefore, they would have to get into the milk while it was being handled, after it had left the condensing pan. This, together with the fact that the usual practice of the manufacturers to prevent this type of spoilage is to raise the percentage of sugar in the milk, led to the following assumptions: First, that factory contaminations, such as deposits from the packing boxes of pasteurizers or of cooling vats, and

water from equipment and floor, might contain the organism. Second, that if high sugar content stopped its growth, normal sweetened condensed milk, diluted slightly, ought to allow the organism, if present, to grow.

On the basis of these two assumptions, a quantity of fresh condensed milk containing 73.5 per cent total solids and 43 per cent sucrose was procured. This was divided into four parts, which respectively were diluted to approximately 73, 70, 65 and 60 per cent total solids by the addition of 5 cc. of factory contamination and sterile water in the required amounts. The factory contamination was a mixture of material removed from the packing box of the pasteurizer and of water taken from a broken place in the floor of the department creamery during its operation. To this was added a small amount of slime from an old skim-milk cheese. In mixing the samples, dishes were used which had previously been exposed to the air of the laboratory for several days. The standardized and inoculated mixtures were placed in sterile bottles. These were incubated at 30°C. for eight weeks, and were examined at the end of two, three and eight weeks.

These examinations showed that the samples containing 65 and 60 per cent total solids developed thickening after two weeks of incubation. The 70-per cent sample showed thickening at three weeks, and the 73-per cent sample at eight weeks. In every instance the thickening was accompanied by the fruity odor and the high acidity.

Bacterial examination showed that all samples contained large numbers of coccus forms. Ten cultures were isolated from the four samples which will hereafter be referred to as series S.

Several tests were made with the ten cultures of this series and the results are recorded in table 1. While each culture does not appear in every lot of milk, at least duplicate determinations were made. The check sample for each lot of milk is recorded at the bottom of the table for reference. A plus sign indicates that thickening or fruity odor was present, and a minus sign shows its absence. Where a question mark is found slight changes were observed, but not enough to warrant their being called positive.

From these results it is evident that the only cultures showing consistent thickening, together with fruity odor and high acidity were S-4 and S-8. A cultural and morphological study of these two cultures showed that they were similar to those which had been isolated from the sample of thickened condensed milk, as reported under heading (*a*).

Where the thickening organism came from cannot be stated, as the sources of inoculation were several in number. The results experienced with the different batches of milk used for

TABLE 1

*Results of thickening tests with cultures of series S*

(Incubation period, six weeks at 30°C.)

| CULTURE | MILK USED | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lot 1 | | | Lot 2 | | | Lot 3 | | | Lot 4 | | |
| | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid |
| | | | *per cent* | | | *per cent* | | | *per cent* | | | *per cent* |
| S-1 | − | − | 0.334 | | | | − | − | 0.545 | ? | ? | 0.670 |
| S-2 | | | | − | − | 0.416 | | | | − | − | 0.395 |
| S-3 | | | | − | − | 0.307 | | | | − | − | 0.390 |
| S-4 | + | + | 0.503 | | | | + | + | 0.900 | + | + | 0.950 |
| S-5 | | | | − | − | 0.335 | | | | ? | | 0.600 |
| S-6 | | | | | | | − | − | 0.562 | − | − | 0.380 |
| S-7 | | | | ? | − | 0.439 | | | | − | ? | 0.760 |
| S-8 | | | | + | + | 0.510 | + | + | 0.990 | + | + | 0.960 |
| S-9 | | | | ? | − | 0.460 | | | | − | ? | 0.830 |
| S-10 | | | | − | − | 0.364 | | | | − | − | 0.500 |
| S-check | − | − | 0.326 | − | − | 0.363 | + | + | 0.645 | − | − | 0.425 |

the thickening tests showed that the thickening organism might have been present in the original condensed milk. It might also have come from any of the other sources of contamination, or from the air.

As this experiment added two more cultures to the group of thickening organisms, further thickening tests were run with a group of organisms isolated from miscellaneous material.

### *c. From miscellaneous material*

A group of twenty-three coccus forms, series R were isolated from miscellaneous material as described below.

Cultures R-1, R-7, R-8, R-9, R-17, R-18, R-19 and R-20 were isolated from condensed milk samples. Cultures R-2, R-3, R-4, R-5, and R-6 were from powdered milk. Samples of rancid condensed milk furnished cultures R-14, R-15, and R-16. Cultures R-10, R-11 and R-12 were obtained from market milk plates while R-21 and R-22 came from air plates from out doors.

These organisms were tested for thickening power by the same procedure used in studying the cultures of series S, and the results appear in table 2.

TABLE 2

*Results of thickening tests with cultures of series R*

(Incubation period, six weeks at 30°C.)

| CULTURE | MILK USED | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lot 1 | | | Lot 2 | | | Lot 3 | | | Lot 4 | | | Lot 5 | | |
| | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid |
| | | | *per cent* | | | *per cent* | | | *per cent* | | | *per cent* | | | *per cent* |
| R-check | − | − | 0.326 | − | − | 0.363 | + | + | 0.645 | − | − | 0.425 | − | − | 0.324 |
| R-1 | | | | | | | | | | − | − | 0.500 | | | |
| R-2 | + | + | 0.585 | | | | + | + | 1.050 | + | + | 0.760 | | | |
| R-3 | | | | | | | − | − | 0.525 | − | − | 0.385 | | | |
| R-4 | | | | | | | − | − | 0.520 | ? | ? | 0.780 | | | |
| R-5 | | | | | | | | | | − | − | 0.460 | | | |
| R-6 | | | | | | | | | | − | − | 0.490 | | | |
| R-7 | | | | − | − | 0.396 | | | | | | | | | |
| R-8 | | | | ? | | 0.495 | | | | | | | | | |
| R-9 | − | − | 0.347 | − | − | 0.307 | − | − | 0.480 | − | − | 0.500 | | | |
| R-10 | − | − | 0.355 | ? | | 0.440 | | | | | | | | | |
| R-11 | | | | | | | | | | − | − | 0.500 | | | |
| R-12 | | | | | | | | | | − | − | 0.430 | | | |
| R-13 | | | | − | − | 0.306 | − | − | 0.765 | − | − | 0.490 | | | |
| R-14 | + | + | 0.502 | | | | | | | − | − | 0.430 | | | |
| R-15 | − | − | 0.310 | | | | | | | | | | | | |
| R-16 | | | | | | | − | − | 0.535 | − | − | 0.470 | | | |
| R-17 | − | ? | 0.462 | | | | | | | − | ? | 0.550 | | | |
| R-18 | − | − | 0.320 | | | | | | | ? | ? | 0.590 | | | |
| R-19 | − | − | 0.325 | | | | | | | − | − | 0.450 | | | |
| R-20 | | | | | | | | | | − | − | 0.500 | | | |
| R-21 | − | − | 0.330 | | | | | | | − | − | 0.550 | | | |
| R-22 | | | | | | | | | | | | | + | + | 0.459 |
| R-23 | | | | | | | | | | | | | + | + | 0.396 |

A study of the results obtained with series R shows that culture R-2 consistently produced thickening in three tests. Culture R-14 gave positive results in the first test, but failed to do so in the second trial. Cultures R-22 and R-23 were only tested once, as they were isolated almost at the completion of the work, when there was no time for further tests.

TABLE 3

*List of pure cultures, obtained from various collections, used in thickening tests*

| SAMPLE | NAME OF CULTURE | NUMBER | COLLECTION |
|---|---|---|---|
| A-1 | Staphylococcus aureus | 4 | American Museum of Natural History |
| A-2 | Staphylococcus aurantiacus | 348 | |
| A-3 | Staphylococcus epidermidus | 25a | |
| A-4 | Staphylococcus ureae | 464a | |
| A-5 | Staphylococcus candidus | 49a | |
| A-6 | Staphylococcus tetragenus | 209 | |
| A-7 | Staphylococcus candicans | 526 | |
| A-8 | Staphylococcus pyogenes aureus | 4 | |
| A-9 | Staphylococcus pyogenes albus | 163 | |
| A-10 | Micrococcus flavus | 272 | |
| A-11 | Micrococcus citreus | 466 | |
| A-12 | Sarcina lutia | 208 | |
| A-13 | Mixed cultures | 4 + 263 | |
| A-14 | Mixed cultures | 4 + 209 | |
| A-15 | Micrococcus lactis varians | 129 | W. M. Esten |
| A-16 | Micrococcus lactis varians | 130 | |
| A-17 | Staphylococcus creamoris viscosi | | Lister Institute |
| A-18 | Staphylococcus pyogenes albus I | | |
| A-19 | Staphylococcus pyogenes albus | | Army Medical School |
| A-20 | Staphylococcus pyogenes albus | 121 | W. M. Esten |

Considering R-2, R-22, and R-23 as positive thickeners, these cultures were studied and found to be similar to the other thickening organisms isolated. Culture R-2, when grown in milk, showed a slight difference in its rate of peptonization from that shown by the other cultures, but otherwise it was the same.

It is of interest to note that the source of R-2 was a sample of powdered milk, while R-22 and R-23 came from the air.

As seven cultures of the thickening organism had thus far been isolated it was decided to test next a series of cultures of known origin. Pure cultures were accordingly obtained from various collections, as listed in table 3.

TABLE 4

*Results of thickening tests with cultures of series A*

(Incubation period, six weeks at 30°C.)

| CULTURE | MILK USED | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lot 1 | | | Lot 2 | | | Lot 3 | | | Lot 4 | | | Lot 5 | | |
| | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid | Body | Odor | Lactic acid |
| | | | *per cent* | | | *per cent* | | | *per cent* | | | *per cent* | | | *per cent* |
| A-check | − | − | 0.326 | − | − | 0.363 | + | + | 0.645 | − | − | 0.424 | − | − | 0.324 |
| A-1 | − | − | 0.312 | − | − | 0.437 | | | | − | − | 0.400 | | | |
| A-2 | | | | | | | − | − | 0.590 | − | − | 0.400 | | | |
| A-3 | − | − | 0.468 | | | | | | | − | − | 0.460 | | | |
| A-4 | | | | | | | − | − | 0.500 | − | − | 0.440 | | | |
| A-5 | | | | | | | | | | − | − | 0.450 | | | |
| A-6 | − | − | 0.321 | − | − | 0.425 | | | | − | − | 0.390 | | | |
| A-7 | − | − | 0.350 | | | | − | − | 0.460 | − | − | 0.490 | | | |
| A-8 | | | | | | | | | | − | − | 0.400 | | | |
| A-9 | − | − | 0.369 | | | | | | | − | − | 0.950 | | | |
| A-10 | | | | − | − | 0.406 | | | | ? | ? | 0.695 | | | |
| A-11 | | | | − | − | 0.408 | | | | ? | ? | 0.670 | | | |
| A-12 | | | | | | | − | − | 0.515 | − | − | 0.500 | | | |
| A-13 | | | | − | − | 0.574 | | | | − | − | 0.740 | | | |
| A-14 | | | | ? | − | 0.380 | | | | − | − | 0.470 | | | |
| A-15 | | | | | | | | | | | | | − | − | 0.405 |
| A-16 | | | | | | | | | | | | | − | − | 0.324 |
| A-17 | | | | | | | | | | | | | − | − | |
| A-18 | | | | | | | | | | | | | − | − | 0.288 |
| A-19 | | | | | | | | | | | | | − | − | |
| A-20 | | | | | | | | | | | | | − | − | |

These cultures were tested for thickening power as the other cultures had been. Several cultures of Staphylococcus pyogenes albus were obtained and included in this series, which will be referred to as series A. Beside testing pure cultures, mixed cultures were used in A-13 and A-14 to determine whether or not there was any associative action.

In studying the results shown in table 4 it will be noticed that

in not a single instance was thickening produced. The thickening organism may resemble Micrococcus pyogenes albus, as stated by Greig-Smith (1). But the results already given would indicate that this organism is not able to produce thickening in sweetened condensed milk. The writer assumes that Micrococcus pyogenes albus referred to by Greig-Smith was the organism described by Rosenbach, and with which the name Staphylococcus pyogenes albus is used synonymously.

*d. From fluid milk*

Considering fluid milk as a possible source of the thickening organism, several samples of fluid milk were studied. It at once became evident that some cultural characteristic would have to be used to distinguish the possible thickeners from the other organisms.

As the acid peptonization of litmus milk seemed to be a characteristic by which the thickening organisms could be distinguished from other milk organisms, the following procedure was used to isolate them. Milk samples were plated on standard nutrient agar, and incubated from two to four days at 30°C. All the colonies on a plate or on part of a plate were picked out and inoculated into litmus milk tubes. These were incubated at 30°C. for ten days.

In a preliminary study it was found that over 11 per cent of the cultures isolated from samples of fluid milk showed acid peptonization. It at once became evident from these results that if many samples of milk were to be examined and the thickening power of all the members of the peptonizing group tested, some other method would have to be devised than that used in the first part of the work, because of the time and the space required for these determinations.

After experimenting with various different methods, the following procedure was adopted: A sample of commercial sweetened condensed milk was obtained and examined. If it appeared to be normal and the acidity as shown by titration was 0.3 per cent lactic acid or below, it was accepted for use. The condensed milk was transferred to a sterile bottle, weighed,

TABLE 5

*Occurrence of thickening organisms in fluid milk*

Thirty-six samples

| SAMPLE NUMBER | PLATE COUNT PER GRAM | CULTURES ISOLATED | ACID PEPTONIZERS FOUND | THICKENERS FOUND | |
|---|---|---|---|---|---|
| | | | | 50 per cent | 70 per cent |
| | *number* | *number* | *number* | *number* | *number* |
| 15 | 9,600 | 20 | 2 | 0 | 0 |
| 16 | 10,600 | 15 | 0 | 0 | 0 |
| 18 | 2,200 | 11 | 0 | 0 | 0 |
| 19 | 5,200 | 16 | 2 | 0 | 0 |
| 20 | 4,700 | 25 | 2 | 1 | 0 |
| 21 | 14,700 | 18 | 15 | 1 | 0 |
| 22 | 7,000 | 11 | 0 | 0 | 0 |
| 23 | 10,000 | 9 | 0 | 0 | 0 |
| 24 | 24,000 | 16 | 1 | 0 | 0 |
| 25 | 115,000 | 15 | 0 | 0 | 0 |
| 26 | 113,000 | 15 | 11 | 0 | 0 |
| 27 | 34,000 | 13 | 0 | 0 | 0 |
| 28 | 4,200 | 15 | 2 | 0 | 0 |
| 29 | 6,200 | 12 | 7 | 0 | 0 |
| 30 | 52,000 | 14 | 3 | 0 | 0 |
| 31 | 37,000 | 11 | 0 | 0 | 0 |
| 32 | 27,000 | 13 | 3 | 0 | 0 |
| 33 | 13,000 | 15 | 3 | 0 | 0 |
| 34 | 380,000 | 17 | 11 | 0 | 0 |
| 35 | 10,000 | 14 | 1 | 1 | 0 |
| 36 | 12,100 | 14 | 4 | 0 | 0 |
| 37 | 6,700 | 12 | 6 | 0 | 0 |
| 38 | 52,000 | 20 | 1 | 0 | 0 |
| 53 | | 15 | 0 | 0 | 0 |
| 57 | | 17 | 0 | 0 | 0 |
| 34-x | 182,000 | 12 | 1 | 0 | 0 |
| 35-x | 11,000 | 13 | 1 | 0 | 0 |
| 36-x | 16,000 | 14 | 0 | 0 | 0 |
| 37-x | 13,900 | 17 | 0 | 0 | 0 |
| 38-x | 16,900 | 13 | 0 | 0 | 0 |
| 39-x | 80,000 | 9 | 0 | 0 | 0 |
| 40-x | 156,000 | 16 | 0 | 0 | 0 |
| 41-x | 98,000 | 16 | 1 | 0 | 0 |
| 42-x | | 14 | 0 | 0 | 0 |
| 43-x | | 17 | 3 | 1 | 0 |
| 46-x | | 9 | 0 | 0 | 0 |
| Totals..... | | 523 | 80 (15.2%) | 4 | 0 |

and standardized to 50 per cent total solids with sterile water. It was then thoroughly mixed by shaking, and was transferred to sterile test tubes plugged with cotton. Ten cubic centimeters of the condensed milk was placed in each tube.

These tubes were immediately inoculated with approximately 0.5 cc. of a litmus-milk culture of the organism to be tested. The tubes were then incubated at 30°C. for two days in a position similar to that used in slanting agar. Cultures of the thickening organism gave a solid coagulation of the milk, which retained its slant form even when vigorously shaken and jarred. This type of coagulation was considered a positive test.

By this method it was possible to examine cultures obtained from numerous samples of milk from individual patrons as well as the mixed milk in the creamery and city milk samples.

While studying this source, thirty-six samples of fluid milk were studied with results as shown in table 5. Of the 523 cultures isolated, 80 cultures classed as acid peptonizers were inoculated, into 50 per cent, sweetened condensed milk and incubated as previously described. Of this number only four cultures produced thickening. These cultures were then tested in 70 per cent sweetened condensed milk in which they all failed to produce thickening.

Although the thickening organism itself was not found in fluid milk, it is quite evident that organisms that are similar do occur and that these organisms can only be distinguished from the thickeners by the thickening test.

*e. From commercial canned milk*

The first study of commercial canned milk was made on ten samples of common brands. The bacterial content per gram, as shown by the plate method, varied greatly, ranging from 30 to 868,000 per gram. Of 20 cultures of cocci isolated, all, with the exception of three cultures from one sample, were inactive in litmus milk after incubation for two weeks. The three exceptions showed slight coagulation and then very slight peptonization. These results gave the impression that the samples did not contain the thickening organisms. Further study, however

brought out the fact that probably a large percentage of the cultures isolated from these samples were the thickening organisms.

Subsequent to the first study, eleven more samples were obtained and studied. The samples were bought on the market, from the local stores. The cans were disinfected with 5 per cent carbolic acid and dried over a flame before being opened. The contents were then transferred to sterile bottles, and stored in the refrigerator. The percentages of solids and of sucrose were

TABLE 6

*Showing the brands used and the condition of the commercial samples at the time of purchase*

| SAMPLE | BRAND | PERCENTAGE COMPOSITION | | | CONDITION OF SAMPLE | | |
|---|---|---|---|---|---|---|---|
| | | Total solids | Sucrose | | | | |
| | | | Total | In water | Body | Taste | Odor |
| | | *per cent* | *per cent* | *per cent* | | | |
| C-1 | Thrift | | | | O. K. | Slightly rancid | Rancid |
| C-2 | Dairymen's League | | | | O. K. | O. K. | O. K. |
| C-3 | A and P | | | | O. K. | O. K. | O. K. |
| C-4 | Eagle | 72.52 | 42.21 | 60.56 | O. K. | O. K. | O. K. |
| C-5 | Eagle | 72.75 | 40.26 | 59.63 | O.K. | O. K. | O. K. |
| C-6 | Thrift | 73.99 | 44.85 | 63.29 | O. K. | Tallowy | Tallowy |
| C-7 | A and P | 74.23 | 41.46 | 61.69 | O. K. | O. K. | O. K. |
| C-8 | A and P | 74.54 | 44.22 | 63.43 | O. K. | O. K. | O. K. |
| C-9 | Eagle | 72.94 | 42.22 | 61.02 | O. K. | O. K. | O. K. |
| C-10 | A and P | 74.45 | 43.80 | 63.15 | Heavy | Old | O. K. |
| C-11 | A and P | 74.45 | 43.61 | 63.00 | O. K. | O. K. | O. K. |

determined, and the condition of the sample was observed. The results are recorded in table 6.

A bacterial count was made by the plate method. Ten-gram samples were used, and dilutions used were 100, 1000 and 10,000. Standard nutrient agar was the medium employed, and the plates were incubated for four days at 30°C. The acidity was determined by titrating with tenth normal sodium hydroxide, a ten-gram sample diluted with 90 cc. of distilled water. The percentage of acid was reported in terms of lactic acid.

A portion of a plate from each sample was selected, and all the

colonies on that portion were picked and inoculated into both litmus milk and agar slants. Notes were kept concerning the types of colonies that predominated, and it was observed that in most of the samples there appeared colonies that closely resembled the typical colony of the thickening organism as did the agar stroke cultures. However, the litmus-milk cultures did not show acid peptonization.

Meanwhile, a portion of each of the original samples had been incubated at 30°C., in order to show the effect of this temperature. It was observed that at the end of ten days samples C-3 and C-10 had thickened to the point of solidity and had developed the characteristic fruity odor. A study of the litmus milk cultures from sample C-3 showed 19 inert reactions out of 20 cultures, while those from C-10 showed 8 inert out of 9 cultures. This observation, together with the fact that these same cultures produced colonies and growths on agar slants that resembled that of the thickening organisms, showed that for some reason the thickening organism, when isolated from samples of condensed milk, does not always produce acid peptonization in litmus milk.

In order to determine whether or not this might be a different organism from the one previously studied, further observations were made. A number of cultures were invigorated in a 1 per cent sucrose-and-lactose broth. The cultures were transferred daily for two weeks, and then were inoculated into litmus milk. When these cultures were incubated, acid peptonization was produced, as in the cultures of the thickening organism originally studied.

It was evident that the peptonization of litmus milk could not be used as a means of identification on organisms that had been isolated from commercial samples of sweetened condensed milk. The identification of the organism from commercial samples was therefore based on the typical colony produced on agar plates, on the typical growth of the agar slant, and on the ability of the organism to thicken 50 per cent, sweetened condensed milk. These tests were further substantiated by inoculating representative cultures from each sample into 70 per cent condensed milk to test their thickening properties.

Using the same procedure as before, the original samples were again plated and cultures isolated, with the results shown in table 7. Thickening organisms were found in all but four of the samples as purchased. In some of the samples, the organism was present in a practically pure culture. The fact that the body and the acidity of the milk were normal would indicate that the organisms had gained entrance in large numbers during its manufacture. This view is further strengthened by the fact that the samples in which no thickening organisms were found at this time were also very low in bacteria count.

TABLE 7

*Occurrence of thickening organisms in commercial samples at the time of purchase*

| SAMPLE | LACTIC ACID | BACTERIA COUNT PER GRAM | CULTURES ISOLATED | THICKENING ORGANISM FOUND | PERCENTAGE OF ALL CULTURES THAT WERE THICKENERS |
|---|---|---|---|---|---|
| | *per cent* | *number* | *number* | *number* | *per cent* |
| C-1 | 0.550 | 215 | 12 | 0 | 0 |
| C-2 | 0.392 | 351,000 | 10 | 9 | 90 |
| C-3 | 0.401 | 32,400 | 12 | 12 | 100 |
| C-4 | 0.391 | 9,500 | 9 | 8 | 89 |
| C-5 | 0.333 | 140 | 12 | 0 | 0 |
| C-6 | 0.327 | 150 | 14 | 0 | 0 |
| C-7 | 0.333 | 6,300 | 13 | 2 | 16 |
| C-8 | 0.303 | 124,000 | 14 | 9 | 64 |
| C-9 | 0.303 | 30 | 11 | 0 | 0 |
| C-10 | 0.354 | 43,000 | 15 | 14 | 93 |
| C-11 | 0.366 | 5,200 | 13 | 6 | 46 |

Sixty cultures of the thickening organisms were isolated from the samples as they were purchased. After the samples had been held six weeks at 30°C., another examination was made, and seventy-three cultures of the thickening organism were isolated. A part of each sample was diluted to 70 per cent solids, and was held at 30°C. for six weeks. Eighty-three cultures of the thickening organism were then isolated from them. From each of the thirty three samples studied in this way, a representative culture was taken and inoculated into 70 per cent milk, as has been done in the other experiments. The results of this test are given in table 8. More cultures were isolated from the

TABLE 8

*Results of thickening test on 33 representative organisms isolated from commercial samples*

| SAMPLE | CULTURE | BODY | ODOR | LACTIC ACID |
|---|---|---|---|---|
| When purchased | | | | |
| | | | | *per cent* |
| 1 | C-1 | − | − | |
| 2 | C-2 | + | + | 0.531 |
| 3 | C-3 | + | + | 0.585 |
| 4 | C-4 | + | + | 0.540 |
| 5 | C-5 | − | − | 0.315 |
| 6 | C-6 | − | − | 0.297 |
| 7 | C-7 | + | + | 0.585 |
| 8 | C-8 | + | + | 0.396 |
| 9 | C-9 | − | − | 0.301 |
| 10 | C-10 | + | + | 0.565 |
| 11 | C-11 | + | + | 0.576 |
| After being held at 30°C. for six weeks | | | | |
| 1 | I-C-1 | Culture lost | | |
| 2 | I-C-2 | Culture lost | | |
| 3 | I-C-3 | + | + | 0.605 |
| 4 | I-C-4 | ? | ? | 0.387 |
| 5 | I-C-5 | + | + | 0.693 |
| 6 | I-C-6 | + | + | 0.694 |
| 7 | I-C-7 | + | + | 0.630 |
| 8 | I-C-8 | + | + | 0.675 |
| 9 | I-C-9 | + | + | 0.602 |
| 10 | I-C-10 | + | + | 0.630 |
| 11 | I-C-11 | + | + | 0.522 |
| Standardized to 70 per cent solids and held at 30°C. for six weeks | | | | |
| 1 | I-C-1-70 | − | − | |
| 2 | I-C-2-70 | + | + | 0.639 |
| 3 | I-C-3-70 | + | + | 0.666 |
| 4 | I-C-4-70 | + | + | 0.441 |
| 5 | I-C-5-70 | + | + | 0.630 |
| 6 | I-C-6-70 | + | + | 0.621 |
| 7 | I-C-7-70 | + | + | 0.324 |
| 8 | I-C-8-70 | + | + | 0.558 |
| 9 | I-C-9-70 | − | − | |
| 10 | I-C-10-70 | + | + | 0.657 |
| 11 | I-C-11-70 | + | + | 0.600 |

same samples after nine weeks of incubation, making a total of 476 cultures, of which 311 were reported as thickeners.

To ascertain what degree of accuracy could be expected in these determinations, tabulations were made. Thirty cultures were classified, according to observations made on the type of colony and on the growth on agar slants, as follows: 24 positive, 2 questionable, and 4 negative.

When they were tested in 70 per cent milk, the following results were obtained: 23 positive, 1 questionable, and 6 negative. It is obvious from these results, therefore, that the figures given are fairly accurate.

*Summary of isolation experiments*

Isolation of the thickening organism was attempted from five sources: thickened condensed milk (*a*); factory contamination (*b*); miscellaneous material (*c*); fluid milk (*d*); and commercial samples of condensed milk (*e*).

A total of 1054 cultures were studied, and 318 cultures were reported as thickening organisms. A total of 284 thickening tests were run in 70 per cent, sweetened condensed milk. So many cultures were obtained from experiment E that only representative cultures were studied from each sample. These samples were studied in the condition in which they were found at the time of their purchase, and again after being held at 30°C. for six weeks, and, finally, after they had been diluted to 70 per cent total solids and held for six weeks at 30°C. In this way the thickening organism was found in all of the samples except C-1. The thirty-two cultures of the thickening organism recorded in table 9 were considered as representative, and were subsequently studied further.

If we consider the isolation experiments from the view point of the possible source of the organism in the sweetened condensed milk, we obtain the results recorded in table 10.

Of the entire 318 cultures of the thickening organism isolated, 313 came from commercial samples of condensed milk and from the original sample of thickened milk. The rest comprised two cultures isolated from factory contamination, one from powdered milk, and two from the air.

TABLE 9

*List of the thirty-two cultures of thickening organisms studied and their source of isolation*

| CULTURE | ISOLATED FROM |
|---|---|
| T-1 | Samples of thickened condensed milk |
| T-2 | |
| S-4 | Factory contamination |
| S-8 | |
| R-2 | Powdered milk |
| R-22 | Outdoor air |
| R-23 | |
| C-2 | Commercial samples as purchased |
| C-3 | |
| C-4 | |
| C-7 | |
| C-8 | |
| C-10 | |
| C-11 | |
| I-C-3 | Commercial samples incubated 6 weeks |
| I-C-4 | |
| I-C-5 | |
| I-C-6 | |
| I-C-7 | |
| I-C-8 | |
| I-C-9 | |
| I-C-10 | |
| I-C-11 | |
| I-C-2-70 | Commercial samples diluted to 70 per cent solids and incubated |
| I-C-3-70 | |
| I-C-4-70 | |
| I-C-5-70 | |
| I-C-6-70 | |
| I-C-7-70 | |
| I-C-8-70 | |
| I-C-10-70 | |
| I-C-11-70 | |

That condensed milk acts as a differential medium for this organism is very evident. The only specific place where thicken-

ing organisms were found was the air. It is quite possible, indeed, that the organisms in the factory contaminations came also from the air.

If the air is a source of contamination, it is easy to see how condensed milk might become contaminated after manufacture, especially in factories where sanitary conditions are not of the best. If this were true, one would expect to find some correlation between the bacterial content of sweetened condensed milk and the presence of the thickening organism, and a study of these two factors, as shown in table 7, shows that this is the case.

TABLE 10

*Summary of the isolation experiments*

| EXPERIMENT | SAMPLES STUDIED | CULTURES STUDIED | THICKENING ORGANISMS FOUND |
|---|---|---|---|
| | number | number | number |
| Thickened condensed milk (*a*) | 1 | 2 | 2 |
| Factory contamination (*b*) | 4 | 10 | 2 |
| Miscellaneous material (*c*) | | 42 | 3 |
| Fluid milk (*d*) | 46 | 523 | 0 |
| Commercial samples (*e*) | 11 (55) | 476 | 311 |

## DESCRIPTION OF THICKENING ORGANISMS

The thirty-two cultures reported in table 9 were further studied to determine whether they were all the same species of organism.

A complete study of the thirty-two cultures was made, as outlined on the descriptive chart endorsed by the committee on pure-culture study of the Society of American Bacteriologists, in their recent reports (4).

The method here reported for comparison is the brief characterization as given on the chart but with a few additions. The cultural features in litmus milk were believed to be especially important in this work, and therefore were included. The fermentation of glycerine was also added. These additions made a total of seventeen instead of nine figures. The explanation of the numerical values is to be found in the key.

*Key to the brief characterization of cultures*

*Microscopic features:*

Form: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinae; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous.
Spores: 1, central; 2, polar; 3, absent.
Flagella: 1, peritrichic; 2, polar; 3, absent.
Gram stain: 1, positive; 2, negative.

*Miscellaneous biochemical reactions:*

Pathogenicity, and so forth: 1, for man; 2, for animals; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic.
Relation to oxygen; 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe.
Gelatin liquefaction: 1, positive; 2, negative.
In nitrate media: 1, nitrite and gas; 2, nitrite but no gas; 3, neither nitrite nor gas.
Chromogenesis: 1, fluorescent; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, pink; 0, none.

*Carbohydrate reactions:*

Diastatic action: 1, positive; 2, negative.
From dextrose: 1, acid and gas; 2, acid without gas; 3, no acid.
From lactose: 1, acid and gas; 2, acid without gas; 3, no acid.
From sucrose: 1, acid and gas; 2, acid without gas; 3, no acid.
From glycerine: 1, acid and gas; 2, acid without gas; 3, no acid.

*Cultural features in milk:*

Acid: 1, sufficient for curdling; 2, insufficient for curdling; 3, no acid.
Rennet curd: 1, present; 2, absent.
Peptonization: 1, present; 2, absent.

Results of the study of thirty-two cultures of thickeners are recorded in table 11. These results show that there are two, quite distinct groups of organisms. Most of the organisms isolated from the commercial samples of condensed milk neither fermented lactose nor reduced nitrates. They were also inactive in litmus milk. Their ability to ferment lactose could be restored, however, by continued invigoration in lactose broth; but their ability to reduce nitrates was never definitely demonstrated.

It is also interesting to note that neither culture I-C-4 nor culture I-C-7-70 fermented sucrose or lactose, and thus they produced little or no acid in the thickening test, as shown in table 8. However, both of these cultures thickened milk. This might indicate that the thickening process was not due to acid but to a rennin-like enzyme. This possibility is further confirmed by the fact that I-C-4 gave a rennet curd in litmus milk.

Just why there should be this difference in the organisms isolated from commercial milk is hard to explain. It would seem logical that, if the organism uses sucrose instead of lactose when

TABLE 11

*The brief characterization number of the thirty-two cultures of the thickening organisms studied*

| CULTURE | MICROSCOPIC FEATURES | BIOCHEMICAL REACTIONS | CARBOHYDRATE REACTIONS | LITMUS-MILK REACTIONS |
|---|---|---|---|---|
| T-1 | 3-3-3-1 | 4-2-1- -0 | 2-2-2-2- | 1-2-1 |
| T-2 | 3-3-3-1 | 4-2-1- -5 | 2-2-2-2- | 1-2-1 |
| S-4 | 3-3-3-1 | 4-2-1-2-0 | 2-2-2-2-2 | 1-2-1 |
| S-8 | 3-3-3-1 | 4-2-1-2-0 | 2-2-2-2-2 | 1-2-1 |
| R-2 | 3-3-3-1 | 4-2-1-2-0 | 2-2-2-2-2 | 1-2-1 |
| R-22 | 3-3-3-1 | 4-2-1-2-0 | 2-2-2-2-2 | 1-2-1 |
| R-23 | 3-3-3-1 | 4-2-1-2-0 | 2-2-2-2-2 | 1-2-1 |
| C-2 | 3-3-3-1 | 4-1-1-3-0 | 2-2-2-2-2 | 1-2-2 |
| C-3 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| C-4 | 3-3-3-1 | 4-2-1-3-0 | 2-2-3-2-2- | 3-2-2 |
| C-7 | 3-3-3-1 | 4-2-2-3-0 | 2-2-3-2-2 | 3-2-2 |
| C-8 | 3-3-3-1 | 4-1-1-3-6 | 2-2-3-2-2 | 2-2-2 |
| C-10 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| C-11 | 3-3-3-1 | 4-2-2-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-3 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-4 | 3-3-3-1 | 4-2-2-3-0 | 2-2-3-3-2 | 3-1-2 |
| I-C-5 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-6 | 3-3-3-1 | 4-2-2-3-0 | 2-2-3-2-2 | 3-2-2 |
| I-C-7 | 3-3-3-1 | 4-2-2-3-0 | 2-2-3-2-2 | 3-2-2 |
| I-C-8 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-9 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-10 | 3-3-3-1 | 4-2-2-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-11 | 3-3-3-1 | 4-2-2-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-2-70 | 3-3-3-1 | 4-2-1-2-0 | 2-2-2-2-2 | 1-2-2 |
| I-C-3-70 | 3-3-3-1 | 4-2-2-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-4-70 | 3-3-3-1 | 4-2-2-2-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-5-70 | 3-3-3-1 | 4-2-2-3-0 | 2-2-3-2-2 | 3-2-2 |
| I-C-6-70 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-7-70 | 3-3-3-1 | 4-2-1-2-0 | 2-2-3-3-2 | 3-2-2 |
| I-C-8-70 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-10-70 | 3-3-3-1 | 4-2-1-3-5 | 2-2-3-2-2 | 3-2-2 |
| I-C-11-70 | 3-3-3-1 | 4-2-2-3-5 | 2-2-3-2-2 | 3-2-2 |

growing in commercial milk, as shown by Rice and Downs (3), its lactose-fermenting enzyme would gradually become dormant. If this is true, when the organism is removed from the presence of

sucrose, which is its only source of energy, it should appear to be a different organism, and so it does. However, continued growth in the presence of lactose, with no other source of energy present, should cause the lactose-fermenting enzyme gradually to become active again. In fact, this seems to be what happens. But why the nitrates are not reduced to nitrites is a subject for further study.

*Description of variety A*

Index number: 3331-42120-22222-121.

*Morphology.* Cultures studied from growth on plain-agar slants, after incubation for two days at 30°C., appear as cocci. The individuals appear as single cells, pairs, and irregular clusters. The size of the organism varies from 0.4 to 0.5 microns in diameter. The organisms stain readily with all common stains, and no capsule is demonstrated. The gram is positive.

*Temperature relations.* Growth on agar slants and in broth attains its maximum at 30°C. There is a slight growth at 20°C., and at 37°C. growth is a trifle less than at 30°C.

*Agar-stroke culture.* Four days at 30°C. produce a moderate, filiform-to-beaded, raised, and glistening growth. The surface is slightly contoured, with its edges raised above the level of the center. The growth is translucent, without any chromogenesis or odor, and generally viscid in consistency.

*Agar colonies.* The surface colonies incubated four days at 30°C. show moderate growth, circular colonies, and a smooth surface with convex elevation. The edges appear to be beveled, and sometimes the center is depressed. The edge is entire, and the internal structure is amorphous. The diameter of the colonies varies from 0.75 to 1.5 mm. and the color, from translucent to pale white.

*Subsurface colonies.* The subsurface colonies are lens shaped, and approximately 0.5 mm. in length. They have a tendency to appear slightly yellowish or straw colored, but they become whitish when growth breaks through to the surface.

*Gelatin stab.* After fourteen days at 20°C. in plain gelatin, growth appears along the line of the stab. This growth is beaded at the bottom, and infundibuliform. Its liquefaction begins in four days. After sixty days it appears as stratiform to the depth of about 1 mm. This description was found to be variable in the same culture, depending on the age of the culture.

*Gelatin colonies at 20°C. (seven days).* Punctiform colonies appear, with slight depression around them.

*Litmus milk at 30°C.* Type a: An acid curd appears after two days, and a shrinking of the curd, and a reduction of the litmus takes place after about four days. As more time passes, the curd contracts and the whey becomes pink and gives an acid reaction to methyl red. Slight peptonization appears after seven days, but it never becomes complete, even when held a year.

Type b: This type is the same as type a, except that the curd begins to peptonize after two days, instead of contracting, and the form of the curd disappears into a mass of granular substance at the bottom of the tube.

*Fermentation.* Acid is produced without gas from fructose, dextrose, lactose, sucrose, and glycerine. It first appears after one day at 30°C. After from two to four days the broth gives a yellow color with brom phenol blue, indicating an approximate pH value of 3.5 to 3. No acid is produced from raffinose, adonite, dulcite, dextrin, or inulin.

*Oxygen requirements.* When the organism grows in dextrose, lactose, sucrose and glycerine broth, it produces a heavy cloud without any gas in the closed arm as well as in the bowl of the fermentation tube.

*Nutrient broth.* On nutrient broth no surface growth appears except a very slight ring growth at the end of from two to four days. A heavy cloud, with a compact and viscid sediment, results from continued incubation.

*Nitrate reduction.* Nitrates are reduced, in four days, to nitrites, when grown on beef-extract nitrate agar.

*Other tests.* When cultures were grown in peptone water for seven days ammonia and indol were produced. No diastatic action is shown when the colonies are grown on starch-agar plates. No hydrogen sulphide is produced.

*Thermal death point.* A broth culture held at 64°C. for ten minutes was killed, but was not killed at 63°C. for ten minutes.

### *Description of variety B (as isolated from commercial samples of condensed milk)*

Index number: 3331-42235-22322-322.

*Morphology.* The morphology of variety B is the same as that of variety A.

*Agar-stroke culture.* The growth is the same as that of variety A, except that the growth is more rapid. Good growth is obtained in

from four to six hours at 30°C. Some cultures show lemon-yellow chromogenesis, both white and yellow sometimes appearing on the same slant.

*Agar colonies.* These are the same as those of variety A, except for their chromogenesis.

*Gelatin stab.* Liquefaction is produced, as in variety A, but it is slower and sometimes absent.

*Gelatin colonies at 20°C. (seven days).* Punctiform colonies appear and liquefaction is questionable.

*Litmus milk at 30°C.* No change takes place in seven days. In two cultures slight acidity was noted, and coagulation was found after fourteen days.

*Fermentation.* Acid is produced, without gas, in dextrose, sucrose, and glycerine. Lactose was not fermented except when slight acidity appeared in the litmus milk.

*Oxygen requirements.* The growth produces a heavy cloud in closed arm with sucrose, dextrose and glycerine broth.

*Nitrate reduction.* Nitrates are not reduced to nitrites.

*Ammonia production.* A small amount of ammonia is produced in peptone water. The production of indol is questionable. No diastatic action or hydrogen sulphide is produced.

These data seem to further confirm the previous belief that the organisms found were all variations of one type of organism. Probably the Staphylococcus pyogenes albus I of the Lister Institute resembles the particular organism in question as closely as do any of the organisms studied. However, this culture did not thicken condensed milk. The question of whether the thickening organism might not lose its ability to thicken milk after a period of time might be raised. While none of the cultures have been carried in stock for a great length of time, some of them were used for more than three years. These cultures were therefore again tested for thickening at the completion of the investigation, and were found to be as vigorous as ever.

## CONCLUSIONS

The group of organisms found to thicken sweetened condensed milk were all of one general type, of which two distinct varieties were found.

Variety A was obtained from one sample of thickened milk, from factory contamination, from air plates, and from powdered milk. This organism was identical with a culture of Staphylococcus pyogenes albus I. from the Lister Institute collection in London, except that albus would not thicken condensed milk. It probably belongs to the same group that has been described by Conn, Esten, and Stocking (5) under the name Micrococcus lactis albidus. The description given there of "variety B" seems to best describe this thickening organism.

Variety B. composed most of the cultures isolated from sweetened condensed milk. These cultures differed from those of variety A in that they did not reduce nitrates to nitrites. They also failed to ferment lactose. In some instances they showed yellow pigment, and produced no change in litmus milk. This organism corresponds very closely to the Micrococcus lactis albidus referred to as "variety C" by Conn, Esten, Stocking (5).

It is evident that many organisms of common occurrence closely resemble these thickening organisms. They can only be distinguished from the thickeners by the thickening test. While they can be found in many places, as already shown, they are found in greatest numbers in sweetened condensed milk as found on the market. Results indicate that they can also be isolated quite readily from the air.

The cause of the thickening, while not studied from the chemical standpoint, can be explained to some extent. It was found that some of the organisms studied produced a high acidity in condensed milk, but did not thicken it. This would indicate that the production of acid alone is not responsible for the thickening. It was also noted that some of the organisms that were unable to ferment either sucrose or lactose produced thickening, and that they produced a rennet curd in litmus milk.

A summary of all of these observations would seem to indicate that the thickening process is caused by a renninlike enzyme produced by the organism.

Organisms that were able to thicken condensed milk were not found to be common in fluid milk.

The thermal death point of the organism being 63°C. in condensed milk, as reported by Greig-Smith (1), and 64°C. in this work, it is evident that, if it existed in the fluid milk, it would not survive the condensing process.

The bacterial content, determined by the plate method, of sweetened condensed milk shortly after its manufacture is a good indication of the sanitary conditions of the plant in which it was made. It is also an index of the number of thickening organisms one should expect to find. This being true, a sample of milk produced under good sanitary conditions, and therefore having a low bacteria count would be less likely to be subject to this type of spoilage.

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# THE INFLUENCE OF SOME BACTERIAL ENZYMES ON THE HEAT COAGULATION OF MILK*

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## THE HEAT COAGULATION OF MILK

It is well known that milk in which much acid formation by bacteria has taken place will curdle when the milk is heated to boiling. Some preliminary experiments had indicated that not only acid formation by bacteria, but also the production of a rennin-like enzyme might influence the coagulation of milk by heat. Since this heat coagulability is important in the evaporated milk industry it was deemed worthy of further investigation.

Most samples of fresh milk can be heated at a temperature of 140°C. or more for a time without permanent coagulation taking place. Sometimes, however, samples of freshly drawn milk will coagulate on heating to the temperature of 115° to 120°C. at which evaporated milk is sterilized. Sommer and Hart (27) have shown that this coagulability of fresh milk is due to the lack of the proper balance of salts in the milk. An excess of calcium in the milk or an excess of phosphates or citrates over the normal balance of salts makes this milk normal.

While the heat coagulatility of a freshly drawn milk may be due to the relation of the salts in the milk, in an older milk bacterial growth may be an important factor. Hart and Sommer (17) have shown that the original potential acidity of the milk does not determine its heat coagulability, nor does the

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original hydrogen-ion concentration. Acidity increased by the addition of acid or its formation by microörganisms does, however, make this milk more easily coagulble.

Of the bacteria that curdle milk two groups might be made: One which brings about the curdling by means of acid alone, and the other which produces a rennin-like enzyme which takes part in the coagulation. To the first group belong most of the more common milk flora, the Streptococcus lactis group, the Coli-aerogenes group, the Bulgaricus group, and a few less important organisms. The rennin formers that occur in milk belong chiefly to the proteolytic sporeforming rods, mostly of the potato bacillus group; although some of the udder cocci produce rennin.

Experiments by Fay (10) and by Rogers (23) had indicated that the rennin produced by bacterial growth might have an effect on the coagulation of milk by heat.

## BACTERIAL RENNIN

Rennin is the active principle which causes the clotting or coagulation of milk casein, and is a term applied to the enzyme causing the coagulation, whatever its source may be. It was originally thought that only extracts from the stomachs or other animal organs contained rennin, but a multitude of workers have, during the past half century or so, showed the presence of rennin-like substances in simpler animals and in plants, molds, and bacteria.

That these rennins from different sources are not identical is indicated by their different physical and chemical properties. As the animal rennins differ, so do the rennin-like substances from plants and microorganisms differ among themselves from the animal rennins.

The sweet curdling of milk by bacteria has been noted by a number of different workers: Heubner (21), Duclaux (7), Warrington (30), Gorini (12) (13); Conn (6), and others. Of the organisms studied by them only those of the potato bacillus group and closely allied organisms occur commonly in milk.

Although they are generally not of much importance in its decomposition, at times they bring about sweet curdling in the absence of acid formers.

## THEORIES OF RENNIN ACTION

The coagulation or clotting of milk casein can be brought about by three general methods: (1) by action of an acid, (2) by action of a curdling enzyme, commonly called rennin, which may be of animal, plant, or bacterial origin, (3) by the action of heat.

In the coagulation of the casein by means of an acid like lactic acid the free protein is liberated from the original calcium caseinate with the formation of calcium lactate (61). The soluble calcium salt thus formed apparently causes the agglomeration of the small particles of colloidal protein and the subsequent precipitation.

The coagulation by rennin, however, does not find such a simple explanation. Space will not permit a discussion of the various theories of rennin action as advanced by: Hammarsten (15), Bosworth (5), Schryver (25) (26), Duclaux (8), Mellanby (19), Bang (3), and Alexander (1). All of the theories apparently indicate a belief that the rennin is not in itself a clotting enzyme, but acts on the original caseinogen molecule and modifies it in such a way that ordinary salt precipitation takes place. This would apply to rennin or rennin-like enzymes from any source, and would help explain the presence of these enzymes in living tissue where there is apparently no use for a curdling enzyme. To quote Falk (9) the action of the enzyme must be considered as a purely chemical catalytic action. In the case of casein precipitation by rennins, we are merely studying substances which place the milk in such a condition that the casein is insoluble in the presence of the existing combination of substances in solution.

## ISOLATION AND PURIFICATION OF RENNIN

The purification of an enzyme is always a matter of difficulty because there is generally a mixture of enzymes present, and

because the enzymes resemble very much some of the organic compounds that always accompany them. So far as is known, no enzyme has yet been obtained in a very pure state. And when the method of action and the conditions necessary for the action of an enzyme are considered, it is doubtful whether it would be an advantage to have an enzyme in an absolutely pure state. It seems probable that the very process of bringing this organic catalyst down to a chemically pure state would destroy or modify the enzymatic action that is desired.

The argument about the identity of rennin and pepsin, however, has led to numerous attempts to separate and purify rennin and pepsin from commercial calves' rennet, and these methods have been extended to the study of rennin-like substances from plants and microörganisms. Hammarsten (15) (16) has published a number of different methods of separation. General methods of purification of enzymes include precipitation of the enzyme by alcohol, acetone, ammonium sulphate, lead acetate, or magnesium sulphate, accompanied by filtration or dialysis or both. The extraction of enzymes from yeast cells is accomplished by ether or chloroform and heat (21). Enzymes have also been purified by selective adsorption on aluminum hydroxide, Fuller's earth, finely divided carbon, etc., although always a great loss of enzymes has resulted. Reynolds (22) has described a method for the concentration and purification of enzymes by ultrafiltration through collodion membranes. While the above methods are fairly successful in partial purification of rennin from animals' stomachs, they do not seem to be as successful in the purification of bacterial enzymes from milk.

Conn (6) was the first worker to report on the isolation of bacterial rennin from cultures. He first grew the rennin-forming organism in milk, and when it had curdled filtered out the curd by means of a procelain filter. The enzyme was precipitated out with alcohol and redissolved in water. He obtained small amounts of rennin in this way. He then effected a partial separation of the rennin from the pepsin by precipitating the rennin with sodium chloride. He found that the porcelain filter held back much of the enzyme and that for that reason only a weak enzyme could be obtained.

Hata (18) filtered the curd from sweet curdled milk, evaporated the filtrate under reduced pressure and precipitated the enzyme with alcohol. The precipitate was treated with an excess of absolute alcohol and dissolved in water, and the enzyme was precipitated with ammonium sulphate. He obtained a mixture of pepsin and rennin.

## EXPERIMENTAL

### *Influence of milk cultures of bacteria on heat coagulation*

It was with the belief that the rennin produced by bacteria might have a considerable influence upon the heat coagulation of milk that the following work was first undertaken. Since Fay's work (10) was the basis for this belief it was thought advisable to check up his work, to some extent, on the effect of acidity, of rennet curdled milk, and of both on the curdling of milk by heat. In agreement with Fay it was found that:

*a.* Small amounts of acid inhibit heat coagulation over a short range.

*b.* Larger amounts of acid hasten heat coagulation.

*c.* Small amounts of a culture of a sweet curdling organism inhibit heat coagulation over a short range.

*d.* In the presence of little acid the addition of portions of a culture of a sweet curdling organism lowers the acidity at which heat coagulation will take place.

*e.* In the presence of larger amounts of acid this action of the culture is obscured or eliminated.

### *Influence of purified enzymes on heat coagulation*

When this part of the work was first begun it was with the hope that bacterial rennin could be isolated and separated from the other enzymes that might be present, especially the proteolytic ones. This, however, was not successfully accomplished, and all of the extracts of purified enzymes used below contained not only the rennin, but also proteolytic and possibly other enzymes. This was not deemed a great disadvantage, for in a general study of the effect of the sweet curdling organisms upon heat coagulation, the rennin enzyme would not be acting alone but in combination with the other enzymes of the organism.

The comparative strength of the enzyme extracts, isolated as described below, was first measured by Morgenroth's method (20) and later by modifications of this method. In Morgenroth's method a constant amount of enzyme solution is added to varying amounts of fresh milk to which one per cent of chloroform has been added. The tubes are placed at a temperature of from 0° to 5°C. for twelve hours or so, and then placed in a 37°C. water bath. The dilution at which curdling takes place within three or four hours is observed, and this taken as a measure of the strength of the rennin present. By this method very small amounts of curdling enzyme can be detected. The first modification made was the use of skim milk from powdered milk instead of fresh milk, as they were found to be equally efficient, while the skim milk was more constant in composition.

Later it was found that there was enough enzyme present in the extracts to test directly at 37°C., without the overnight incubation at 0°C. With the small tubes used it was found that the 37°C. incubator was as satisfactory as the 37°C. water bath. Observations were made at intervals of an hour up to the fourth or fifth hour. In some of the later work 0.6 per cent phenol was used as an antiseptic in place of 1 per cent chloroform. The phenol was not quite as satisfactory as the chloroform.

Several of the purified bacterial enzymes were used in the comparison of milks for use in the strength tests. Fresh milk-powder skim milk, sterilized (autoclaved) milk powder skim milk, pasteurized skim milk, and fresh whole milk were compared. Fresh milk-powder skim milk and fresh whole milk were found to be equally satisfactory for use with the bacterial rennin. Pasteurized skim milk was not as good, but could be used. The autoclaved milk powder milk was worthless. For use with chymosin or calves' rennet the fresh whole milk was superior to the other kinds.

The first attempts at the isolation and purification of bacterial rennin were made with the use of Conn's original method (6). In agreement with Conn, it was found that when the milk culture of the organism was filtered through Chamberland or Berkefeld filters that most of the enzymes were lost by absorption.

The next method attempted was similar to that used by Gerber (11) in the isolation of rennin from plant tissues. The milk culture of the sweet curdling organism was filtered through filter paper to remove the curdled casein. The filtrate was saturated, with ammonium sulphate. The coagulum, generally a scum, was skimmed off or filtered out and redissolved in a 5 per cent solution of NaCl. The solution was saturated again with ammonium sulphate. The coagulum was removed and placed in a parchment sac where it was dialyzed in running distilled water for several days. The resulting solution or suspension was used as freshly as possible and kept in the ice box until used. This method was fairly successful. Enzyme solutions were prepared which themselves were one-fourth to one-fifth of the original volume of milk culture, and which coagulated milk in dilutions as high as 1:50 or 1:100. If concentrated enzymes had been desired these solutions could have been evaporated under partial vacuum.

Collodion membranes were tried in place of parchment membranes but were not as good. This is explained by Bechhold (4) who found that rennin is absorbed in great amounts by collodion filters. It was also noted by Vandervelde (28) that a cellulose membrane was satisfactory for dialysis of a rennin containing solution.

The casein was removed from the original milk cultures with difficulty, particularly when an old culture was used in which considerable digestion had taken place. Attempts were made to remove this casein by various methods without the removal or destruction of the enzymes. The addition of acid was first tried. It was found that a very limited amount of acid could be used without much harm to the enzyme. Acetic and lactic acids were used, and if not over 0.5 per cent acid was added the enzyme was weakened but little. When the addition of acid was combined with heat, however, still less acid could be added.

The use of heat alone up to 50° to 55°C. gave a stronger enzyme solution than without heat. A temperature of 60°C. weakened the enzyme perceptibly. When heat and acid were combined very little acid could be used, for 0.25 per cent of acetic acid gave a weaker enzyme.

Lead acetate and calcium acetate were combined with heat with weakened enzymes as a result. The use of ethyl sulphuric acid was unsuccessful. Avery and Cullen (2) used bile in getting enzymes from the pneumococcus. This was not successful with the rennin enzymes of the sweet curdlers.

Most of the sweet curdling organisms used had an optimum growth temperature of 37°C. The optimum temperature for the production of rennin, however, was at lower temperatures of from 20° to 28°C.

More rennin was always found in an old culture, and one in which considerable digestion had taken place. Little enzyme was ever obtained from a culture that had freshly sweet-curdled. The rennin could be isolated from cultures which never curdled the milk but only digested it. The same observation was made as that by Gorini (14) and by Conn (6) that some of the organisms, which sweet-curdle milk when first isolated, lose that property on cultivation and have increased proteolytic power.

In attempts at the isolation and purification of the rennin from some 70 bacterial cultures in milk the following procedure was finally adopted as the most successful. The sweet curdling organism was inoculated into sterile milk and the milk incubated at 25°C. for five to six days. This culture was heated at 50°C. for fifteen minutes. If the casein separated out well, no acid was added, otherwise about 0.2 per cent glacial acetic acid was added upon removal of the milk from the 50°C. water bath. The culture was filtered through filter paper. The filtrate was saturated with crystalline ammonium sulphate; and the coagulum that formed was skimmed off and redissolved in water. This solution was saturated with ammonium sulphate, and the coagulum was skimmed off and placed in a parchment thimble (Schleicher and Schull). Dialysis was carried on for two days in running distilled water. If quantitative comparisons of the enzymes were to be made, measured amounts of culture were used and the final enzyme solutions were made up to definite volume. The enzyme solutions were kept in the ice box. Attempts to sterilize them by various methods failed.

Cultures of the organisms in nutrient broth were allowed to

grow for several weeks to insure considerable autolysis of the cells. Little enzyme could be isolated from the cultures.

Direct dialysis of the milk serum gave a fairly strong enzyme solution, which was probably in a very impure state.

*Heat coagulation tests with purified enzymes*

When the purified enzyme solution was added directly to milk in varying concentrations and the milk heated immediately at either 100° or 120°C. in no case was an effect on heat coagulation observed. Thirty trials were made with enzymes from ten different organisms with the same result in all experiments. All of these enzymes were strong enough so that one part of enzyme would curdle thirty parts of milk within three hours. When the enzyme has not been allowed to act on the milk, then, it has no effect upon heat coagulation. Indeed, a temperature of 50°C. for fifteen minutes would weaken the purified enzyme, while 60°C. would amost entirely destroy it. The same enzyme in the original milk culture was more heat resistant. This has been observed by other workers.

In order for the enzyme to have any effect upon heat coagulation, the enzyme must have been allowed to act upon the milk for some time. This is indicated by experiments of which the following is an example.

Two enzyme solutions $Q_2B$ and 10E, had been previously tested for strength. One part of $Q_2B$ would coagulate fifty parts of milk within four hours. Solution 10E would coagulate about thirty parts of milk in the same time. One part of either enzyme to four parts of milk would not coagulate at 100°C. for thirty minutes.

After incubation at 37°C. for different lengths of time, the varying dilutions of the enzymes in milk were removed from the incubator, the amount of coagulation noted and the tubes then heated at 100°C. for thirty minutes. The results are shown in table 1.

The results of the above experiment show that the heat apparently carries to completion the coagulation begun by the enzyme, if that action has gone far enough.

Similar results are obtained when the tubes are heated in the autoclave at 137°C. instead of 100°C.

The optimum temperature for the action of these rennin-like enzymes is about 40°C. or slightly higher. At 37°C., then, the temperature used in the above experiments, favorable conditions obtained for the action of the enzyme on the milk. If the enzyme is allowed to act on the milk at room temperature, however, there is still a marked influence on heat coagulation. When, for instance, the two enzymes used in the previous experiment were allowed to act on milk at room temperature for an hour, the only coagula-

TABLE 1

| ENZYME | TIME AT 37°C. | COAGULATION DILUTION | |
|---|---|---|---|
| | | After 37°C incubation | After thirty minutes more at 100°C. |
| | *minutes* | | |
| $Q_2B$ | 15 | 1:5 | 1:10 |
| 10E | 15 | Negative | 1:5 |
| $Q_2B$ | 30 | 1:10 | 1:15 |
| 10E | 30 | 1:5 | 1:10 |
| $Q_2B$ | 45 | 1:15 | 1:30 |
| 10E | 45 | 1:5 | 1:15 |

tion at that time was with 10E in a 1:5 dilution. When the tubes of milk were autoclaved, coagulation took place as follows:

10E—1:5, 10, 15, 20, 30 = ++; 1:40 = negative
Pr —1:5, 10, 15 = ++; 1:20 = negative

Similar results were obtained with other enzyme solutions.

Even at the ice box temperature, 10°C., the enzyme has its effect, although no curdling is noted when the tubes are removed from the ice box. The following experiment with the same enzyme illustrates this point.

Dilutions of the two enzymes in milk were made; and the tubes were placed in the ice box at 10°C. for ten hours. At the end of this period there was no indication of curdling in any of the tubes. A set of tubes with each enzyme was immediately

autoclaved at 20 pounds pressure for ten minutes. Coagulation took place as follows:

10E—1:5, 10, 15, 20, 30 = ++; 1:40, 50 = partial
Pr —1:5, 10 = ++; 1:15 = partial

As a check upon the ten hour action of the enzymes at 10°C., two sets of tubes were also placed at 37°C. and the rate and amount of coagulation noted. It will be observed that this procedure is really the same as Morgenroth's method for detecting very small amounts of rennin. As was expected the action was rapid and soon came to completion. Within fifteen minutes 10E had curdled at a 1:15 dilution, and Pr at 1:5. After an hour 10E had coagulated in a 1:40 dilution and Pr in 1:15.

The above experiments have shown that the bacterial rennin can act upon the milk in such a way as to favor heat coagulation, even though no outward sign of its action is to be observed. The rate of this enzyme action is dependent upon the temperature. As is the case with all enzyme actions and most chemical reactions, an increase in temperature increases the rate of action up to the optimum temperature. The rate of action of the bacterial rennins studied has been roughly proportional to the amounts of enzyme used.

Since the presence of calcium is necessary for coagulation by animal rennet, and since calcium also apparently plays a part in ordinary heat coagulation of milk, it was thought advisable to study the effect of calcium and citrates upon the action of bacterial rennin.

Comparable to the way in which the presence of calcium helps the action of animal rennin at ordinary temperatures (50) was the effect of calcium on the action of bacterial rennin. The addition of small amounts of calcium acetate (M/12) hastened the curdling perceptibly at room temperature and at 37°C. The addition of sodium citrate had the opposite effect, that is it inhibited enzyme action. These results would indicate that the action of bacterial rennin might not only be like the action of animal rennin, but might also be similar to that of the coagulation by heat due to a lack of the proper balance of milk salts. If this were true then the enzyme action should have an effect that

would be additive to the effect of an excess of calcium in the milk salts. Experiments indicate that this is true.

In table 2 is shown the effect of the addition of calcium acetate and of sodium citrate in amounts proportional to the amount of enzyme added. M/12 calcium acetate was used, and M/4 sodium citrate; and one part of the salt solution was added for each part of enzyme. Thus a dilution of 1:30 would mean one part of calcium acetate solution to 29 parts of milk solution and also one part of enzyme to 29 parts of total solution. The salts and enzymes were added to varying amounts of milk and the tubes allowed to stand for an hour at room temperature. The amount of curdling, if any, was observed at this time; and then

TABLE 2

*Effect of calcium acetate or sodium citrate plus enzyme upon heat coagulation*

| SERIES NUMBER | ENZYME | SALT ADDED | DILUTIONS AT WHICH COAGULATION TOOK PLACE | |
|---|---|---|---|---|
| | | | At 22°C. for one hour | At 125°C. for ten minutes |
| I | 10E | | 1:5 | 1:30 |
| II | Pr | | Negative | 1:50 |
| III | 10E | Ca acetate | 1:15 | 1:40 |
| IV | 10E | Na citrate | Negative | 1:15 |
| V | Pr | Ca acetate | 1:10 | 1:30 |
| VI | Pr | Na citrate | Negative | 1:10 |
| VII | | Ca acetate | Negative | 1:15 |
| VIII | | Na citrate | Negative | Negative |
| Controls | | | Negative | Negative |

the tubes were heated in the autoclave at 125°C. for ten minutes.

It will be noted in table 2 that the effect of the addition of calcium acetate on the action of the enzymes at room temperature is also shown, for coagulation has taken place at higher dilutions after one hour where the calcium salt was added. The important thing to be noted in the table, however, is how the effect of enzyme plus calcium acetate is practically an addition of the separate effects. On the other hand, the addition of sodium citrate had an inhibitive effect upon the heat coagulation.

The additive effect of the enzyme action and the action of calcium is again shown in table 3. In this experiment the amount of calcium acetate in the milk was constant, i.e., it was added to

the whole lot of milk, while only the enzyme was varied in concentration. The amount of calcium acetate added was below the amount which would cause heat coagulation. If, now, heat coagulation took place at dilutions greater than those at which the enzyme action alone was effective, then it could be concluded that the action of enzyme plus calcium was additive. Heat coagulation was tested in the autoclave, where the tubes were heated at 20 pounds pressure for ten minutes.

As is shown in table 3, heat coagulation took place at twice as great a dilution when calcium acetate was present as when the enzyme was alone, even when the amount of calcium acetate present was not enough to cause heat coagulation.

A series of experiments was run to show that the influence of an excess of calcium was effective whether the enzyme had been

TABLE 3

*Additive effect of calcium acetate and enzyme (at 125°C)*

| SERIES | ENZYME | DILUTIONS AT WHICH COAGULATION TOOK PLACE | |
|---|---|---|---|
| | | Without calcium acetate | With calcium acetate |
| I | 10E | 1:20 | 1:50(plus) |
| II | Pr | 1:20 | 1:50 |
| III | BSR | 1:10 | 1:20 |
| VI | $Q_3$ | 1:10 | 1:20 |
| Controls | | Negative | Negative |

acting at lower temperatures or at higher ones. Enzyme solutions from four different organisms were used. Two of the solutions, 10Ea and Pra. were comparatively strong and would coagulate milk in dilutions of 1:40 and 1:30, respectively, after two hours at 37°C. The other two enzyme solutions, $Q_3$a and BSRa, were weaker and coagulated milk in dilutions of 1:15 and 1:10 respectively.

A series of dilutions of the enzyme solution was made in milk-powder milk. A second similar series was treated with calcium acetate solution so that there was added one part of M/12 calcium acetate to each part of enzyme solution. In a third series the enzyme was added, the milk was treated with calcium acetate solution, so that the strength of the added calcium acetate was M/240. This was not enough to cause coagulation by heat.

The three series were incubated at the temperature desired and then tested for heat coagulation.

In table 4 is shown how calcium acetate plus enzyme action affect the heat coagulability of the milk, when incubation has been at ice box temperature (11°C.) for ten hours.

It will be noted in table 4 that in all cases the effect of the enzyme and the calcium acetate is additive. The expression of the strength of added calcium acetate at the heat coagulation

TABLE 4

*Influence of calcium acetate and enzymes on heat coagulation*

Incubation at 10° for ten hours

| ENZYME | TREATMENT WITH $Ca$ ACETATE | DILUTION AT WHICH COAGULATION TOOK PLACE | | STRENGTH OF $Ca$ ACETATE AT HEAT COAGULATION DILUTION |
|---|---|---|---|---|
| | | At 10°C. for ten hours | At 120°C. for ten minutes | |
| 10Ea | a* | Negative | 1:30 | |
| 10Ea | b | 1:10 | 1:50 | M/600 |
| 10Ea | c | Negative | 1:50 | M/240 |
| $Q_1a$ | a | Negative | 1:10 | |
| $Q_1a$ | b | Negative | 1:15 | M/180 |
| $Q_1a$ | c | Negative | 1:20 | M/240 |
| Pra | a | Negative | 1:10 | |
| Pra | b | 1:10 | 1:30 | M/360 |
| Pra | c | 1:5 | 1:50 | M/240 |
| BSRa | a | Negative | 1:5 | |
| BSRa | b | Negative | 1:15 | M/180 |
| BSRa | c | Negative | 1:15 | M/240 |
| Control | Like b | Negative | 1:5 | M/60 |
| Controls | | Negative | Negative | |

* Treatment "a" = no calcium acetate added. Treatment "b" = one part of M/12 calcium acetate to one part of enzyme. Treatment "c" = strength of calcium acetate is M/240 in all dilutions of enzyme.

dilution does not give an absolute measurement of the amount of effective calcium in all cases. With enzyme 10Ea, for instance, there seems to be a wide variation in the amount of added calcium acetate at the point of heat coagulation. The dilutions were made, however, only through 1:50; and coagulation would probably have proceeded farther. This would also hold for the third treatment with Pra. It will be noted, however, that when only calcium acetate is added to the milk that the presence of

M/60 calcium acetate is necessary to bring about heat coagulation while in the presence of enzyme 10Ea heat coagulation took place with M/600 calcium acetate present.

When incubation took place at room temperature or at 37°C. the results were similar to those at ice box temperature.

The effect of enzyme and of calcium acetate is similar, then, whether the temperature of incubation is high or low. At higher temperatures the action of the enzyme is more rapid and would come to completion sooner than at lower temperatures. The results of this work has indicated that a definite quantity and strength of enzyme can accomplish only a certain amount toward making the milk heat coagulable. Even though the time of enzyme action may not have been sufficient to bring about coagulation at the temperature of incubation, the enzyme has acted upon the milk in such a way that the coagulation by heat will take place at as high a dilution as that at which coagulation would have taken place at the optimum temperature for enzyme action. In all of the above experiments controls kept at 37°C. have borne out this statement. The added calcium acetate has had a double effect on heat coagulation. It has not only per se made the milk more heat coagulable, but it has made the enzyme more effective.

As a matter of comparison the effect of chymosin or calves' rennet on heat coagulation was studied. The chymosin seems to act exactly like the bacterial rennin. As is well known, soluble calcium aids the action of the chymosin. When the chymosin had been allowed to act upon the milk for a short time, heat coagulation took place at that dilution at which coagulation at 37°C. would have taken place if the reaction had been allowed to go to completion.

It has been shown that the presence of soluble calcium in small amounts helps or hastens the action of bacterial rennin. That the presence of small amounts of acid would produce a similar effect is to be expected, for the acid would also serve to form soluble calcium salts. Experiments with six different enzymes have shown that the addition of small amounts of lactic acid hastens the curdling at 37°C. by bacterial rennin and carries the curdling out to higher dilutions.

## STUDIES OF ORGANISMS USED

A detailed study of the more important organisms used in the above experiments was made, according to the methods endorsed by the Society of American Bacteriologists. Most of the organisms belong to the potato bacillus group, but although they answer to the general characteristics of that group, they will not fall into any of the species mentioned by Bergey. It was not deemed necessary to record in this paper complete descriptions of the organisms. All of these potato bacillus-like organisms were spore forming rods, motile, and gram positive. All formed chains, mostly short. With the exception of B. subtilis they were facultative toward oxygen with a tendency toward aerobic. All sweet curdled milk with subsequent digestion, more or less complete. Some lost their ability to sweet curdle milk after a period of cultivation in the laboratory. All varied among themselves in certain details, and all were isolated from milk which ha d sweet curdled.

In addition to these spore forming rods, three non-spore forming saprophytes were used. Enzymes from B. pyocyaneus, B. fluorescense liquefaciens, and B. prodigiosus were purified and studied.

## DISCUSSION

The practical application of the influence of bacterial rennin on heat coagulation of milk would, of course, be in the evaporated milk industry. The influence of acid upon heat coagulation has long been known. Hart and Sommer (58) have shown the effect of an excess or lack of calcium in the milk salts upon heat coagulation. The results of the above experimental work have indicated that bacterial rennin also has an effect upon heat coagulation. It has been noted that the cause of heat coagulation in all three cases is dependent on the calcium salts in the milk. In coagulation by means of acid the release of calcium as a salt of the acid probably plays an important part. The heat coagulation of freshly drawn milk is generally due to an excess of soluble calcium. And the heat coagulation of milk which has been exposed to the action of bacterial rennin has

been shown to be connected with the amount of soluble calcium in the milk.

The action of any one of the three above factors may be sufficient to bring about heat coagulation of milk upon sterilization. But since their action is similar, the combined action of any two or all three of the factors would be still more effective.

The application of these results to the evaporated milk industry is evident. Night's milk is rarely delivered to a condensery before the following morning. If bacterial growth has taken place in the milk before cooling, or if it takes place during the night due to insufficient cooling, bacterial rennin may be produced and allowed to act on the milk during the night, even at low temperatures in a way similar to that in the Morgenroth method described above. The development of a slight amount of acid will aid in the action. Or if after preheating and evaporation, some of the spore-forming, rennin-producing rods start to grow and produce rennin, then there will be a long time for its action on the evaporated milk when the milk is cooled and held over night for canning and sterilization the next morning. These spore-forming rods would after the preheating and evaporation processes be freed of competition with the common acid forming organisms which do not form spores.

The thermophilic organisms which produce a rennin-like enzyme may have an opportunity to function. The temperature of the vacuum pan would be favorable to their growth; and there would be time for the action of their enzymes when the evaporated milk is held over night, as shown above; the low temperature of storage would still permit the action of the rennin to take place, so that coagulation would take place on sterilization in the can the following day.

Some of the organisms used in this work grow very rapidly at favorable temperatures; sweet curdling has resulted within eight to ten hours at times.

The production of rennin-like enzymes by bacteria which occur in milk, then, is a factor which should be considered in the coagulation of a milk on sterilization.

SUMMARY

1. The results of the first part of the work where only milk cultures of the sweet curdling organisms were used can be summed up as follows:

*a.* Small amounts of acid inhibit heat coagulation over a small range.

*b.* Larger amounts of acid increase heat coagulation.

*c.* Small amounts of a milk culture of a sweet curdling organism inhibit heat coagulation over a small range.

*d.* Larger amounts of a milk culture of a sweet curdling organism hasten heat coagulation.

*e.* In the presence of little acid the addition of portions of a milk culture of a sweet curdling organism lowers the acidity at which heat coagulation will take place.

*f.* In the presence of larger amounts of acid this action of the culture of a sweet curdling organism is obscured or eliminated.

2. The results of the second part of the work when the rennin-like enzymes of certain bacteria were isolated and purified are as follows:

*a.* The rennin-like enzymes of sweet curdling organisms were isolated and purified to some extent by methods described.

*b.* Bacterial rennin is destroyed by a temperature of 60°C. for fifteen minutes.

*c.* The purified bacterial rennin has no effect upon heat coagulation when the milk is heated immediately upon addition of the rennin.

*d.* When the rennin has been allowed to act on the milk for a time, the heat coagulability of the milk is increased.

*e.* The rate of this action of the rennin is dependent on the concentration or strength of the enzyme present.

*f.* The rate of action of the rennin is dependent on the temperature at which the enzyme is allowed to act. A temperature of 40° to 43°C. is the optimum for most rennins. An increase in temperature up to the optimum temperature hastens rennin action. Action goes on at a slow rate at temperatures as low as 10°C.

*g.* The addition of acid in small amounts accelerates the rennin action.

*h.* The presence of soluble calcium salts in small amounts accelerates the rennin action.

*i.* The presence of citrates in small amounts retards the rennin action.

*j.* The effect of acid plus rennin upon heat coagulation is additive.

*k.* The effect of soluble calcium salts plus rennin upon heat coagulation is additive.

*l.* A definite amount of curdling enzyme can cause heat coagulability only in a limited amount of milk.

*m.* This limit of influence is the same whether the rennin is allowed to complete its action at its optimum temperature or autoclave temperatures are applied after the action is started.

*n.* Chymosin resembles the bacterial rennins in its influence upon the heat coagulation of milk.

*o.* Bacterial rennin may be an important factor in the coagulation of milk upon sterilization in the condenseries. The rennin may work in conjunction with acid in the milk or an excess of soluble calcium.

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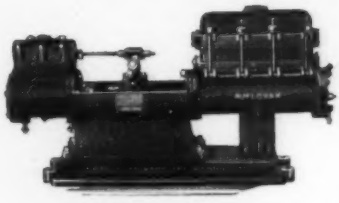

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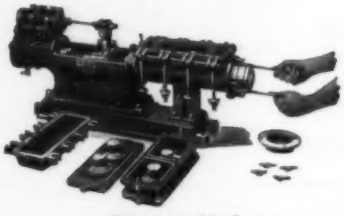

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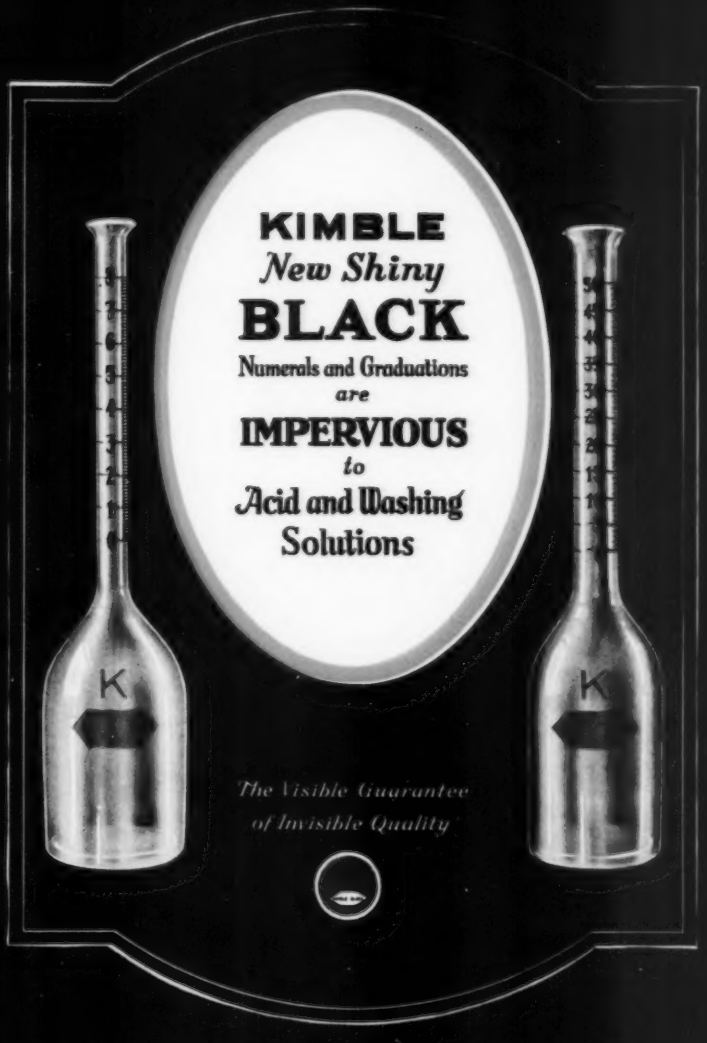
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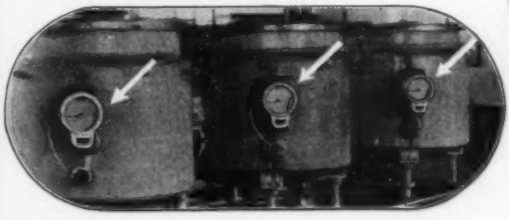

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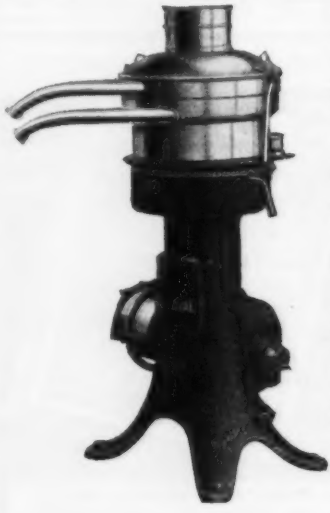

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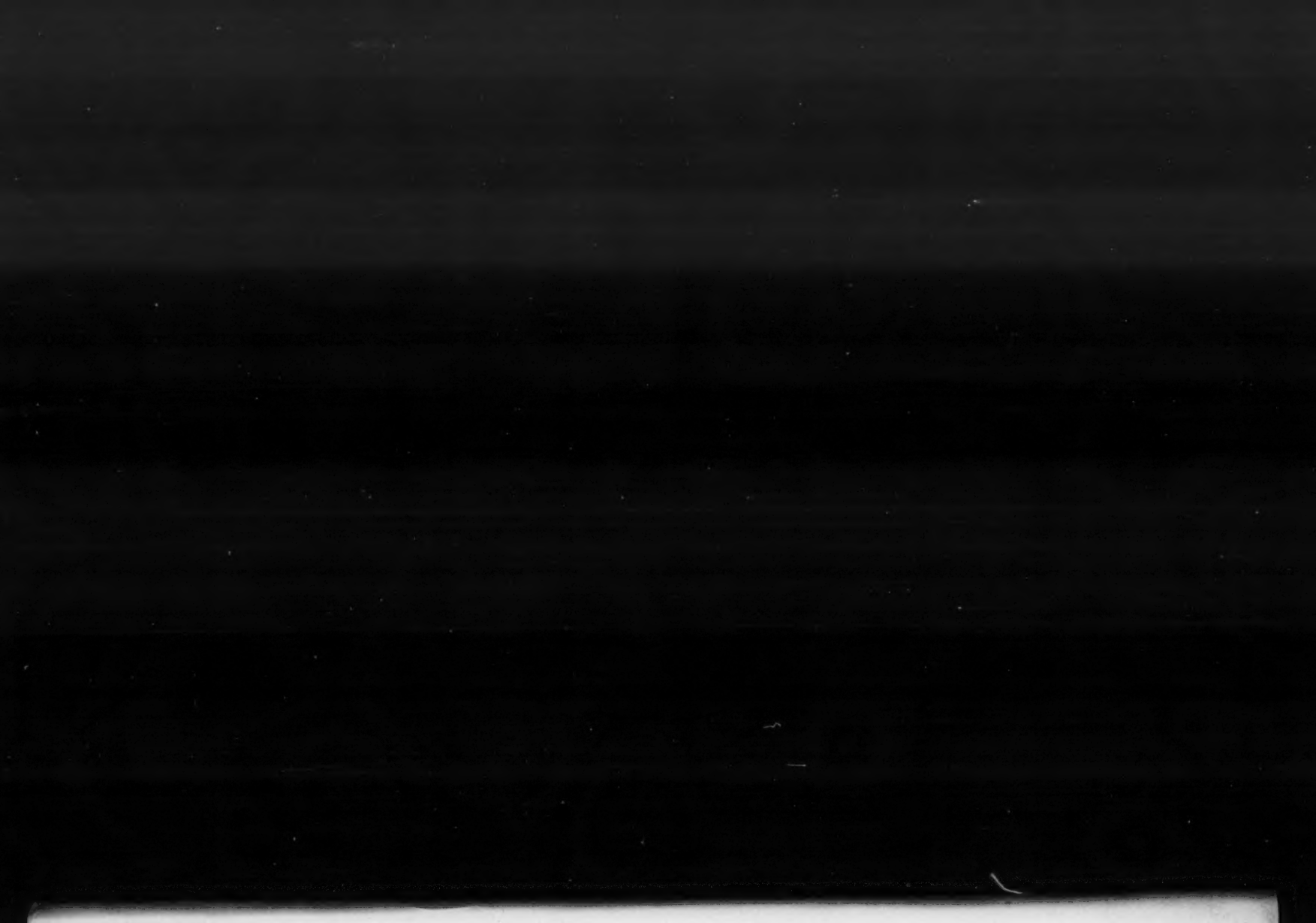

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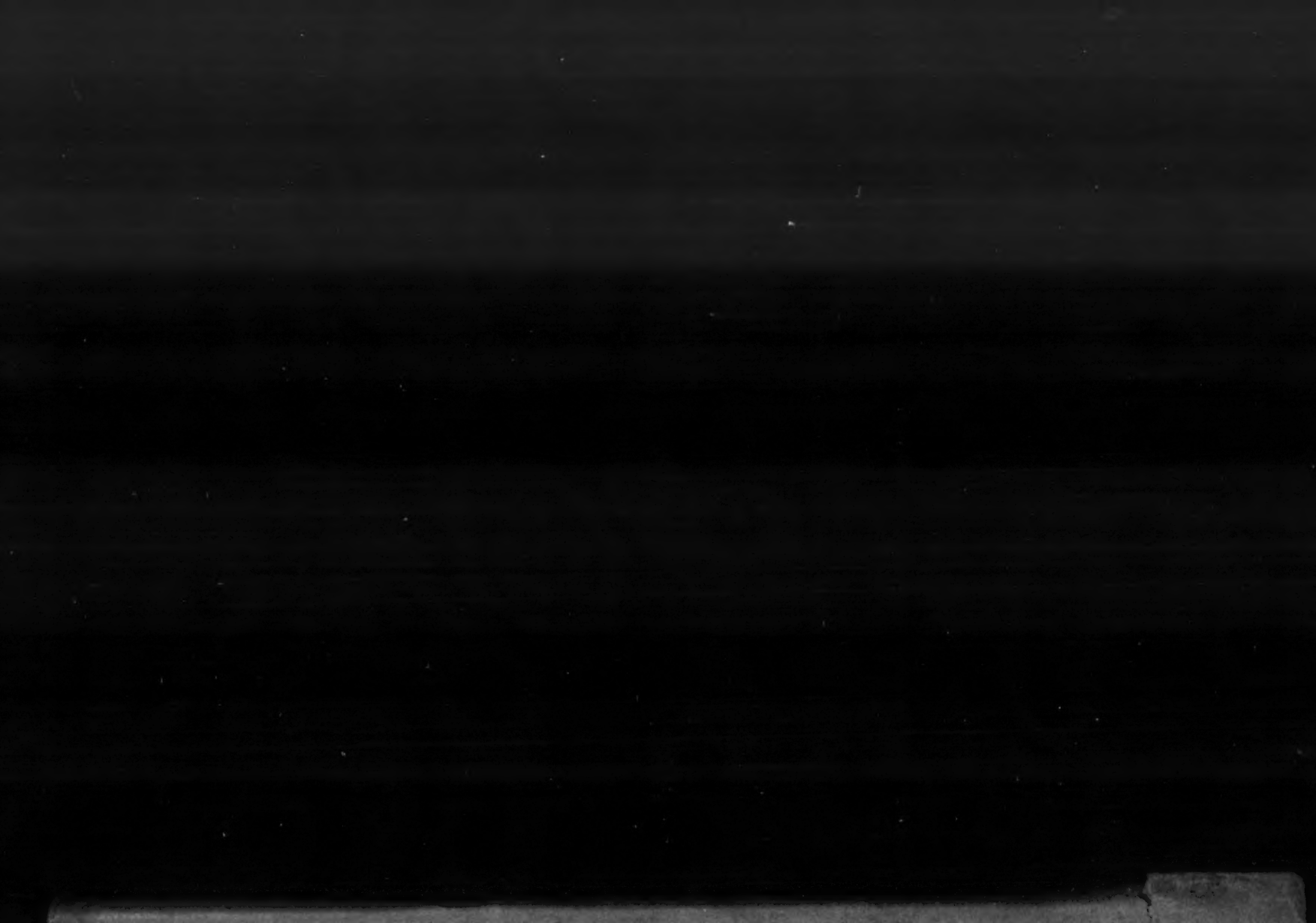
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